THE INTERACTIONS AMONG β2-GLYCOPROTEIN I, NATURAL ANTICOAGULANTS, AND COMPLEMENT: SIGNIFICANCE FOR THE ANTIPHOSPHOLIPID SYNDROME

by

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ABSTRACT

Antiphospholipid syndrome (APS) patients are predisposed to recurrent thrombosis and miscarriages. Elevated levels of complement activation have also been demonstrated. A major antigenic target of antiphospholipid autoantibodies (aPL) is β2-glycoprotein I (β2GPI). Impairments of anticoagulation by aPL have been demonstrated; however, the mechanism is unclear. Also, the in vivo functions of β2GPI in the coagulation and complement systems remain to be established. Therefore, the effects of aPL on the interactions among β2GPI, anticoagulation, and complement system were studied. β2GPI interacted with two anticoagulant proteins, activated protein C (APC) and protein S. β2GPI inhibited total APC activity by competing with APC for the phospholipids. However, β2GPI potentiated APC specific activity on phosphatidylethanolamine and phosphatidylserine. Therefore, β2GPI appeared to inhibit as well as to enhance APC. β2GPI also interacted with protein S and C4b-binding protein (C4BP), a complement regulator. C4BP and protein S circulate as a complex in blood. Analyses of the interactions among β2GPI, protein S, and C4BP showed that oxidized surface-bound β2GPI prevented protein S binding to C4BP by competing for the β-chain on C4BP. Also, β2GPI interacted with protein S when β2GPI was not occupied by C4BP. Thus, β2GPI may have a role in maintaining the free protein S level. Besides C4BP, β2GPI also interacted with another complement protein, mannose-binding lectin (MBL). MBL is a complement activator. Their interactions led to weak in vitro complement activation. Interactions of oxidized surface-bound β2GPI with APC and protein S were inhibited by aPL. Also, aPL inhibited the total APC activity on a phosphatidylserine surface by increasing β2GPI deposition. However, this was
accompanied by an increase in APC specific activity. Therefore, aPL appeared to inhibit as well as enhance anticoagulation activity. Antiphospholipid antibodies inhibited C4BP and enhanced MBL binding to β2GPI. These observations suggest that aPL can promote complement activation. This possibility was further supported by the observation of in vitro complement activation by aPL in patients' plasma. Therefore, β2GPI may normally interact with APC, protein S, C4BP, and MBL to regulate anticoagulation and complement activation. However, aPL may interfere with these protein interactions, and thereby disrupt the anticoagulation mechanism and elevate complement activation.
# TABLE OF CONTENTS

Abstract .......................................................................................................................... ii

Table of Contents .......................................................................................................... iv

List of Tables ................................................................................................................ xi

List of Figures ............................................................................................................... xii

List of Appendix Figures ............................................................................................ xiv

Abbreviations .............................................................................................................. xv

Acknowledgements ....................................................................................................... xviii

Dedication ..................................................................................................................... xix

Chapter 1 Introduction................................................................................................. 1

1.1 Antiphospholipid Antibodies .............................................................................. 1

1.1.1 History ............................................................................................................ 1

1.1.2 Antiphospholipid Antibodies in Antiphospholipid Syndrome .................. 2

1.1.3 Laboratory Tests for Antiphospholipid Antibodies .................................. 3

1.1.4 Formation and Characteristics of Antiphospholipid Antibodies ............ 4

1.1.5 Effects of Antiphospholipid Antibodies on Hemostasis ......................... 5

1.2 Antiphospholipid Syndrome .............................................................................. 6

1.2.1 APS Clinical Manifestations ...................................................................... 6

1.2.2 Classification Criteria for APS ................................................................... 8
Chapter 2  Materials and Methods

2.1  Reagents ................................................................. 41
2.2  Purification of MBL ...................................................... 42
2.3  Normal Human Plasma Preparation .................................. 42
2.4  Normal Human Serum Preparation .................................... 42
2.5  Blood Samples ............................................................ 43
2.6  Detection of Anti-Cardiolipin and Anti-β2GPI Using ELISA ...... 43
2.7  Isolation and Characterization of IgG Pools ....................... 44
2.8  Specificities of IgG Pools .............................................. 44
2.9 125I-Radiolabeling of Proteins ............................................................................. 44

2.10 Effects of Co-Incubating β2GPI and Protein S on APC Activities in
Phosphatidylserine-Coated Wells ........................................................................ 45

2.11 Effects of β2GPI on APC Activities in Phosphatidylcholine-,
Phosphatidylethanolamine-, Phosphatidylserine-Coated Wells ......................... 46

2.12 Interactions of APC, Protein C, Protein S, C4BP, C4BP β, and MBL with
ELISA Plate-Bound β2GPI Under Different Conditions ....................................... 46

2.13 Effects of High Binding ELISA Plate-Bound β2GPI on APC Activities .......... 48

2.14 Kinetics and Equilibrium Dissociation Constants of APC, Protein C, Protein S,
C4BP, MBL, and BSA Interactions with β2GPI ....................................................... 48

2.15 Competitive ELISA For Determining Anti-Protein C Antibody Specificity For
APC and Protein C .................................................................................................. 49

2.16 Effects of Anti-β2GPI Sepharose Beads and β2GPI-Sepharose Beads on
Plasma Protein S Clotting Activity ....................................................................... 50

2.17 Effects of aPL IgG on APC Activities in the Presence of β2GPI and Protein S
in PS-Coated Wells ............................................................................................... 51

2.18 Effects of aPL IgG on β2GPI Binding to PS-Coated ELISA Wells ................ 51

2.19 Effects of aPL IgG on APC, Protein S and C4BP Binding to Activated Platelets
in the Presence of β2GPI ....................................................................................... 52

2.20 Binding of 125I-β2GPI and 125I-Protein S to Activated Platelets in the Presence
of aPL .................................................................................................................... 53

2.21 ELISA To Detect Antiphospholipid Antibody-Mediated Complement
Activation ............................................................................................................... 53
Chapter 3  Effect of β2-Glycoprotein I on the specific activity of Activated Protein C In The Presence Of Aminophospholipids ........................................... 55

3.1 Effects of β2GPI and Protein S on APC Activity on Phospholipid Surface..... 55

3.2 Activity of APC Bound to PC-, PE-, and PS-Coated Wells in the Presence of β2GPI.................................................................................................................. 59

3.3 Effect of Fluid Phase β2GPI on APC and Protein S Binding to Oxidized ELISA Plate-Bound β2GPI in the Absence of Phospholipids............................... 61

3.4 Activity of β2GPI-Bound APC........................................................................ 64

3.5 Inhibition of APC Binding to β2GPI by Protein S ........................................... 68

3.6 Equilibrium Dissociation Constant Determinations for APC, Protein C, Protein S and BSA Binding to β2GPI................................................................. 68

3.7 Comparison of Protein C and APC Binding to β2GPI APC Using ELISA...... 70

3.8 Summary ......................................................................................................... 73

Chapter 4  β2GPI Interactions with C4BP and Protein S..................................... 75

4.1 Analysis of C4BP and β2GPI Interaction in the Presence of Protein S......... 75

4.2 Equilibrium Binding Constants .................................................................... 82

4.3 Effects of β2GPI-Depletion From Plasma Using Anti-β2GPI-Sepharose Beads on Functional Protein S Levels ......................................................... 82

4.4 Effects of Sepharose-coupled β2GPI on Functional Protein S Level......... 84

4.5 Summary ......................................................................................................... 84
Chapter 5 Effects of aPL on β2GPI Interactions with APC, Protein S, and C4BP.. 87

5.1 Characteristics and Specificities of IgG Pools.............................................. 87
5.2 Inhibition of APC, Protein S, and C4BP Binding to β2GPI by aPL IgG pool..90
5.3 Effects of aPL on APC Activity as Measured by Chromogenic Substrate S-2366..................................................................................................................... 90
5.4 aPL Increases β2GPI Binding to Phospholipid Surface ................................ 95
5.5 Binding of APC, Protein S, and C4BP to Activated Platelets in the Presence of aPL and β2GPI ................................................................. 97
5.6 Binding of β2GPI to Activated Platelets in the Presence of aPL IgG ........... 98
5.7 Summary........................................................................................................... 100

Chapter 6 Inhibition of C4b-Binding Protein (C4BP) and Mannose Binding Lectin (MBL) Binding to β2GPI by Antiphospholipid Antibodies......... 101

6.1 Effect of C4b on C4BP Binding to β2GPI....................................................... 101
6.2 MBL Binding to β2GPI in the Presence of Calcium, EDTA and N- acetylmannosamine ......................................................................................... 103
6.3 Binding of MBL to β2GPI-Bound aPL.......................................................... 103
6.4 Equilibrium Dissociation Constant Determinations for MBL Binding to β2GPI ........................................................................................................... 106
6.5 Complement Activation by Antibody-β2GPI Complex .................................. 108
6.6 β2GPI-Mediated Complement Activation.................................................. 110
6.7 Correlations of Complement Activation Level to Antiphospholipid Levels.. 110
6.8 Specificity and Sensitivity of Antiphospholipid ELISAs Relative to Complement Activation ELISA ................................................................. 114
LIST OF TABLES

Table 1. Preliminary Classification Criteria for Definite Antiphospholipid Syndrome. .. 9

Table 2. Equilibrium Dissociation Constant Determinations for APC, Protein C, and Protein S Binding to β2GPI. ............................................................................................................ 71

Table 3. Characteristics of aPL IgG Pool. ........................................................................ 88

Table 4. Binding of Activated Protein C, Protein S and C4BP to Platelets as Detected by Fluorescence Flow Cytometry is not Affected by the Presence of aPL. .......... 99

Table 5. Characteristics of Serum Samples. .................................................................... 112

Table 6. Correlation (r) of Complement Activation to Antiphospholipid Level .......... 113

Table 7. Specificity and Sensitivity of Antiphospholipid ELISAs Relative to Complement Activation ELISA ......................................................................................... 115
LIST OF FIGURES

Figure 1. Primary structure of human β2GPI ............................................................. 11
Figure 2. Scheme of Tissue Factor Pathway of Coagulation and Activated Protein C Anticoagulation System ................................................................. 21
Figure 3. The Complement System ........................................................................ 27
Figure 4. Regulation in the Complement System ................................................... 31
Figure 5. Hydrolysis of Chromogenic Substrate S-2366 by PS-Bound APC .............. 56
Figure 6. Specific Activity of PS-Bound APC in the Presence of β2GPI and Protein S .. 57
Figure 7. Specific Activity of phospholipid-bound APC in the presence of β2GPI in PC-, PE-, and PS- Coated Wells ............................................................... 60
Figure 8. Interaction Between β2GPI and APC ....................................................... 62
Figure 9. Interaction Between β2GPI and Protein S ............................................... 65
Figure 10. Activity of β2GPI-Bound APC .............................................................. 67
Figure 11. Inhibition of APC Binding to β2GPI by Protein S ..................................... 69
Figure 12. APC and Protein C Binding to β2GPI ..................................................... 72
Figure 13. Competitive ELISA For Determining Anti-Protein C Antibodies Specificities For APC and Protein C ................................................................. 74
Figure 14. Interaction Between β2GPI and C4BP ..................................................... 76
Figure 15. Interaction Between β2GPI and C4BP is dependent on the β-subunit of C4BP ........................................................................................................... 79
Figure 16. Inhibition of Protein S Binding to β2GPI by C4BP .................................... 80
Figure 17. Functional protein S levels in β2GPI-depleted plasma ............................. 83
Figure 18. Functional protein S levels in plasma treated with β2GPI-Sepharose. .......... 85
Figure 19. Specificity of the aPL pool for β2GPI. .................................................. 89
Figure 20. Inhibition of APC, protein S, and C4BP binding to β2GPI by aPL. .......... 91
Figure 21. Inhibition of APC, protein S, and C4BP binding to β2GPI by aPL. .......... 92
Figure 22. Activity PS-Bound APC in the Presence of aPL β2GPI and Protein S. .... 93
Figure 23. aPL Increases β2GPI Binding to Phospholipid Surface. ....................... 96
Figure 24. Effect of C4b on C4BP binding to β2GPI. ............................................... 102
Figure 25. MBL Binding to β2GPI. ................................................................. 104
Figure 26. Increased Binding of MBL to β2GPI in the Presence of aPL IgG. ........... 107
Figure 27. Rabbit Anti-Human β2GPI Mediated C5b-9 Generation. ....................... 109
Figure 28. β2GPI-Mediated Complement Activation ............................................. 111
Figure 29. β2GPI Increases the Specific Activity of APC in the Presence of
Aminophospholipid. ......................................................................................... 118
Figure 30. β2GPI Interactions with C4BP and Protein S...................................... 128
Figure 31. Effects of aPL on β2GPI interaction with protein S-C4BP complex. ...... 144
Figure 32. Effects of aPL on APC Activity. ....................................................... 145
LIST OF APPENDIX FIGURES

Appendix Figure 1. Purity of β2GPI, APC, protein S, C4BP, C4BP-β, and MBL........ 179
Appendix Figure 2. Effects of APC Iodination on APC Activity in PS-Coated Wells.. 180
Appendix Figure 3. Dissociation Constant Determination for APC-β2GPI Interaction .......................................................... 181
Appendix Figure 4. Dissociation Constant Determination for Protein C-β2GPI Interaction .......................................................... 182
Appendix Figure 5. Dissociation Constant Determination for Protein S-β2GPI Interaction .......................................................... 183
Appendix Figure 6. Dissociation Constant Determination for C4BP-β2GPI Interaction ............................................................... 184
Appendix Figure 7. Dissociation Constant Determination for MBL-β2GPI Interaction ............................................................... 185
Appendix Figure 8. Effects of Iodination on APC-, protein C-, protein S-, C4BP-, and MBL- Binding to β2GPI. ........................................ 186
# Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
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<tbody>
<tr>
<td>APC</td>
<td>activated protein C</td>
</tr>
<tr>
<td>aCL</td>
<td>anticardiolipin</td>
</tr>
<tr>
<td>aPL</td>
<td>antiphospholipid antibodies</td>
</tr>
<tr>
<td>APTT</td>
<td>activated partial thromboplastin time</td>
</tr>
<tr>
<td>APS</td>
<td>antiphospholipid syndrome</td>
</tr>
<tr>
<td>B2GPI</td>
<td>β2-glycoprotein I</td>
</tr>
<tr>
<td>BCA</td>
<td>bicinchoninic acid</td>
</tr>
<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
</tr>
<tr>
<td>C4BP</td>
<td>C4b-binding protein</td>
</tr>
<tr>
<td>C4BP-B</td>
<td>C4b-binding protein without β-chain</td>
</tr>
<tr>
<td>CL</td>
<td>cardiolipin</td>
</tr>
<tr>
<td>CRP</td>
<td>C-reactive protein</td>
</tr>
<tr>
<td>DAF</td>
<td>decay-accelerating factor</td>
</tr>
<tr>
<td>DIC</td>
<td>disseminated intravascular coagulation</td>
</tr>
<tr>
<td>dRVVT</td>
<td>dilute Russell’s viper venom time</td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>ELISA</td>
<td>enzyme-linked immunosorbent assay</td>
</tr>
<tr>
<td>FITC</td>
<td>fluorescein isothiocyanate</td>
</tr>
<tr>
<td>GAL</td>
<td>galatose</td>
</tr>
<tr>
<td>Gla</td>
<td>gamma(γ)-carboxylated glutamic acid</td>
</tr>
<tr>
<td>GVB&lt;sup&gt;2+&lt;/sup&gt;</td>
<td>VBS, 0.15 mM CaCl&lt;sub&gt;2&lt;/sub&gt;, 1 mM MgCl&lt;sub&gt;2&lt;/sub&gt;, 0.1 % gelatin, pH 7.4</td>
</tr>
</tbody>
</table>
GVB-EDTA  VBS, 20 mM EDTA, 0.1 % gelatin, pH 7.4
HRP  horseradish peroxidase
ICAM-1  intracellular adhesion molecule-1
KCT  kaolin clotting time
LA  lupus anticoagulant
Lp(a)  lipoprotein (a)
MBL  mannose-binding lectin, mannan-binding lectin
MCP  membrane cofactor protein
MHTB  modified HEPES Tyrode’s Buffer
(10 mM HEPES, 137 mM NaCl, 2.7 mM KCl, 0.4 mM NaH$_2$PO$_4$, 0.1% dextrose, 0.2% BSA, pH 6.5)
MHTB-Ca  MHTB, 2.5 mM CaCl$_2$, pH 7.4
MIRL  membrane inhibitor of reactive lysis
NAM  N-acetyl-mannosamine
NHlG  normal human IgG
NHP  normal human plasma
NHS  normal human serum
PC  phosphatidylcholine
PE  phosphatidylethanolamine
PS  phosphatidylserine
RbIgG  rabbit IgG
RCA  regulation of complement activation
RT  room temperature

xvi
<table>
<thead>
<tr>
<th>Acronym</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>SAP</td>
<td>serum amyloid P</td>
</tr>
<tr>
<td>SCR</td>
<td>short consensus repeat</td>
</tr>
<tr>
<td>SLE</td>
<td>systemic lupus erythematosus</td>
</tr>
<tr>
<td>TBS</td>
<td>tris-buffered saline, (10 mM Tris, 150 mM NaCl, pH 7.4)</td>
</tr>
<tr>
<td>TBS-Ca</td>
<td>TBS, 2.5 mM CaCl$_2$, pH 7.4</td>
</tr>
<tr>
<td>TBS-CaM</td>
<td>TBS, 2.5 mM CaCl$_2$, 5% w/v skim milk, pH 7.4</td>
</tr>
<tr>
<td>TBS-EDTAM</td>
<td>TBS, 10 mM EDTA, 5% w/v skim milk pH 7.4</td>
</tr>
<tr>
<td>TBS-TCa</td>
<td>TBS, 0.05% Tween 20, 2.5 mM CaCl$_2$, pH 7.4</td>
</tr>
<tr>
<td>TBS-TEDTA</td>
<td>TBS, 0.05% Tween 20, 10 mM EDTA, pH 7.4</td>
</tr>
<tr>
<td>TCA</td>
<td>trichloroacetic acid</td>
</tr>
<tr>
<td>TM</td>
<td>thrombomodulin</td>
</tr>
<tr>
<td>TMB</td>
<td>3,3',5,5'-tetramethylbenzidine</td>
</tr>
<tr>
<td>VBS</td>
<td>veronal buffered saline (1.8 mM sodium barbitone, 1.56 mM sodium barbituric acid, 145 mM NaCl, pH 7.4)</td>
</tr>
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DEDICATION

To my mother Irene, father Chee Wing, and brother Kenneth.
CHAPTER 1 INTRODUCTION

1.1 Antiphospholipid Antibodies

1.1.1 History

Despite the name, antiphospholipid antibody associated with antiphospholipid syndrome does not bind directly to phospholipid. It owes its name to its reactivity in assays containing the phospholipids used for detecting what were believed to be “true” antiphospholipid antibodies. These “true” antiphospholipid antibodies were first identified in association with syphilis infections. They were “phospholipid” antibodies that bound directly to phospholipid. These antibodies were detected by serologic test for syphilis (STS) using saline extract of syphilitic liver as antigen (Wassermann et al., 1906). The Venereal Disease Research Laboratory (VDRL) test, which contained cardiolipin (CL) as the major antigen, was later developed for antiphospholipid antibody detection (Pangborn, 1941). Thus, these antibodies are also known as anticardiolipin (aCL). However, it became apparent later that these tests also detected anticardiolipin antibodies not associated with syphilitic infection. Individuals without evidence of syphilitic infection sometimes tested positive for STS. These cases were initially named biological false positive-serologic tests for syphilis (BFP-STS) (Davis, 1944). Some of these individuals were also found to have systemic lupus erythematosus (SLE) (Moore and Lutz, 1955). Since some of these anticardiolipin antibodies also exhibited in vitro anticoagulant activity, they were named lupus anticoagulants (LA) (Feinstein and Rapaport, 1972). However, the name lupus anticoagulant is clearly a misnomer because these antibodies are not confined to SLE patients (Triplett and Brandt, 1988) and the
prevalent clinical manifestation is thrombosis rather than a bleeding disorder (Bowie et al., 1963). These investigations together led to the realization of the existence of antiphospholipid unrelated to syphilitic infection and the later discovery of the association of anticardiolipin and LA with antiphospholipid syndrome (APS).

1.1.2 Antiphospholipid Antibodies in Antiphospholipid Syndrome

Anticardiolipin and LA are the two main clinical groups of antiphospholipid antibodies and are characterized by two different assay methods. Anticardiolipin is identified by ELISA and LA is detected by clotting assays. Although they are normally described as anti-phospholipid antibodies, their binding to phospholipid is actually mediated by protein cofactors.

Anticardiolipins are antibodies that react with cardiolipin-binding proteins. The major antigen for anticardiolipin was identified to be β2-glycoprotein I (β2GPI) by three groups independently in 1990 (Galli et al., 1990; McNeil et al., 1990; Matsuura et al., 1990). Therefore, anticardiolipins are actually anti-β2GPI antibodies. However, not all anticardiolipin antibodies are directed against β2GPI. Other antigenic targets of anticardiolipin and antiphospholipid antibodies have been identified, albeit less commonly. Most of these antigens share the phospholipid-binding characteristic of β2GPI and they include prothrombin (Bevers et al., 1991), protein C (Pengo et al., 1996; Oosting et al., 1993), protein S (Pengo et al., 1996; Oosting et al., 1993), annexin V (Sammaritano et al., 1992), thrombomodulin (Ruiz-Arguelles et al., 1989), kininogen (Sugi and McIntyre, 1995), thrombin-antithrombin complex, C4b-binding protein, and lipopolysaccharide binding protein (Arvieux et al., 1999). Therefore, strictly speaking,
anticardiolipin antibodies are a group of protein-dependent antiphospholipid antibodies, which includes anti-β2GPI.

LAs are antibodies that can prolong the clotting time of coagulation assays (Shapiro, 1996; Triplett, 1996). The major coagulation antigen for LA was identified to be prothrombin (Bevers et al., 1991; Fleck et al., 1988; Edson et al., 1984; Bajaj et al., 1983). LA specific for β2GPI have also been described. This is known as anticoagulant-type A. Anti-β2GPI without LA activity is referred to as anticoagulant-type B (Galli et al., 1992). Therefore, even though anticoagulant, anti-β2GPI, LA and aPL are classified into different groups, they are closely related antibodies whose activity may overlap.

1.1.3 Laboratory Tests for Antiphospholipid Antibodies

Anticardiolipin antibodies are routinely detected by two different ELISAs. One has negatively charged phospholipid, usually cardiolipin, coated to the ELISA plate, and uses fetal calf serum that contains β2GPI as diluent of plasma samples (Gharavi et al., 1987). The other one has β2GPI, one of the major target antigens of anticoagulant, directly coated to an oxidized ELISA plate (Roubey et al., 1995; Matsuura et al., 1994). The former ELISA will detect antibodies specific for β2GPI as well as other phospholipid-binding serum proteins because of the use of fetal calf serum as sample diluent. In contrast, the latter ELISA can only detect antibodies specific for β2GPI.

Coagulation assays useful for detecting LA include activated partial thromboplastin time (APTT) sensitive to LA, kaolin clotting time (KCT), and the dilute Russell's viper venom time (dRVVT). A prolonged APTT clotting time suggests the
presence of LA. However, a mixing study (1:1 mix of normal and patient’s plasma) needs to be performed to ensure that the APTT prolongation is not caused by a coagulation factor deficiency. The presence of a putative LA has to be confirmed by the neutralization of its inhibitory activity when phospholipid concentration is increased (Exner et al., 1991). KCT and dRVVT are also effective in detecting LA. In addition, KCT is especially sensitive in detecting prothrombin-specific LA while dRVVT is sensitive for β2GPI-specific LA (Galli et al., 1998; Galli et al., 1995).

1.1.4 Formation and Characteristics of Antiphospholipid Antibodies

Antiphospholipid antibodies (aPL) can be generated by immunization of mice with different antigens. Induction of aPL in mice has been achieved by immunization with heterologous β2GPI (Gharavi et al., 1992). Immunization with synthetic viral and bacterial β2GPI-like peptides also induced aPL production (Gharavi et al., 1999). Immunization of mice with phosphatidylethanolamine (PE) in the hexagonal II phase also induced aPL generation with lupus anticoagulant activity (Rauch and Janoff, 1990). A study has shown that the formation of antiphospholipid antibodies in mice is mediated by T cells (Blank et al., 1995). Recently, the generation of anti-β2GPI antibodies in humans was shown to be β2GPI-specific, requiring class II molecules, CD4+ T cells, and antigen presenting cells (Visvanathan and McNeil, 1999). These two studies both support the participation of cellular immunity in APS.

Different subclasses of IgG have different effector functions (Schur, 1987). Studies of aPL IgG subclasses in human showed that IgG2 is the most prevalent among the four subclasses (Sammaritano et al., 1997; Arvieux et al., 1994; Levy et al., 1990;
Gharavi et al., 1988). Since IgG2 is a weak complement activator compared to IgG3 (40x) and IgG1(6x) (Hamilton, 1987), aPL IgG has been believed not to have an important role in complement activation (Arvieux et al., 1994).

1.1.5 Effects of Antiphospholipid Antibodies on Hemostasis

Antiphospholipid antibodies have numerous effects on hemostasis. These include inhibition of factor XII autoactivation by anti-β2GPI in the presence of β2GPI (Schousboe and Rasmussen, 1995), inhibition of prothrombin activation by aPL (Goldsmith et al., 1994), and inhibition of factor X activation by LA (Shi et al., 1993). These studies showed in vitro anticoagulation effects of aPL. Interestingly, several studies also suggested that aPL has procoagulant effects. These studies include the potentiation of factor X activation by anticardiolipin by impairing β2GPI inhibitory effect (Shi et al., 1993), shortening of prothrombin time by anti-β2GPI with LA activity (Pengo et al., 1999), and the induction of tissue factor expression on monocytes by anticardiolipin (Reverter et al., 1996; Kornberg et al., 1994). Elevated expression of tissue factor on APS patients’ monocytes has also been observed (Cuadrado et al., 1997). The anticoagulation mechanism also appears to be a target of aPL as aPL have been reported to inhibit activated protein C (APC)-mediated inactivation of factor Va (Galli et al., 1998; Oosting et al., 1993; Malia et al., 1990) (Marciniak and Romond, 1989).

Various associations between aPL and platelet function have been reported. Patients with aPL have increased urinary secretion of the thromboxane metabolite thromboxane B₂ in urine, reflecting platelet activation (Forastiero et al., 1998; Martinuzzo et al., 1993; Lellouche et al., 1991; Arfors et al., 1990). Also, aPL can
promote in vitro thromboxane metabolite formation (Lellouche et al., 1991) (Robbins et al., 1998; Leung et al., 1996; Martinuzzo et al., 1993). These reports suggest platelet-activating properties for aPL.

Activation of endothelial cells by anti-β2GPI is associated with the expression of E-selectin, vascular cell adhesion molecule-1 (VCAM-1), intracellular adhesion molecule-1 (Simantov et al., 1995), expression of endothelial leukocyte adhesion molecule-1 (Del Papa et al., 1995), secretion of interleukin-6 and 6-keto-prostaglandin F$_{1α}$ (Del Papa et al., 1997), expression of cyclooxygenase-2 (Habib et al., 1995), and up-regulation of adhesion molecules, increased leukocyte adherence, and interleukin-1β secretion (Del Papa et al., 1997). Endothelial cell activation may yet be another thrombogenic mechanism in APS.

1.2 Antiphospholipid Syndrome

1.2.1 APS Clinical Manifestations

The more commonly seen clinical manifestations of antiphospholipid syndrome (APS) include recurrent venous thrombosis, recurrent arterial thrombosis, thrombocytopenia and recurrent fetal loss (Harris et al., 1986; Harris et al., 1983). Neurological, dermatological, hematological, and cardiopulmonary complications associated with APS are also widely reported.

The most frequent form of thrombosis is venous thrombosis of the deep veins (Alarcon-Segovia et al., 1989). Venous thrombosis in other vascular beds such as the renal veins (Nochy et al., 1999; Piette et al., 1994) and the hepatic portal vein (Pelletier et
Arterial thrombosis occurs less frequently than venous thrombosis (Harris, 1994; Sammaritano et al., 1990). Transient ischemic attack is a common arterial presentation (Trimble et al., 1990). Thrombocytopenia occurs in about 15% to 20% of patients with APS (Cuadrado et al., 1997; Rosove and Brewer, 1992; Alarcon-Segovia et al., 1992; Picillo et al., 1992). Obstetric complications include recurrent spontaneous abortion and recurrent fetal loss (Branch et al., 1985).

Neurological manifestations usually present as chorea (Cervera et al., 1997; Asherson et al., 1987), Guillain Barré syndrome (Chaleomchan et al., 1990), or Sneddon’s syndrome (Kalashnikova et al., 1990). Dermatological manifestations include livedo reticularis, leg ulcers, necrotizing purpura, and peripheral gangrene (Asherson and Cervera, 1993; Stephens, 1991; Alegre et al., 1989). Other hematological manifestations include hemolytic anemia (Hsu et al., 1997; Vandenberghe et al., 1996; Sthoeger et al., 1993) and leukopenia (Vila et al., 1999; Deleze et al., 1989). Cardiopulmonary complications include valvular lesion (Kaplan et al., 1992) and pulmonary hypertension (Badui et al., 1995; Asherson et al., 1990).

APS can occur in patients with another underlying disease or without other complications. APS without any underlying disease is classified as primary antiphospholipid syndrome (Asherson, 1988) while APS associated with other diseases, such as systemic lupus erythematosus (SLE) or other autoimmune diseases, is classified as secondary antiphospholipid syndrome. Approximately 40% of SLE patients possess antiphospholipid antibodies (Love and Santoro, 1990). Of these SLE patients with aPL, about 40% develop thrombosis. In comparison, only 12% of SLE patients without aPL develop thrombosis.
1.2.2 Classification Criteria for APS

Diagnosis of APS has to satisfy both clinical and laboratory criteria. The preliminary criteria for classification of APS proposed by the International Consensus Workshop in 1998 is listed in Table 1 (Wilson et al., 1999).

1.2.3 Management of APS

Anticoagulation therapy with long term use of warfarin to achieve an International Normalized Ratio (INR) > 3 with or without low dose aspirin (75 mg) was found to be effective in preventing recurrent venous and recurrent arterial thrombosis (Khamashta et al., 1995; Rosove and Brewer, 1992). Corticosteroid treatment is effective for thrombocytopenia (Khamashta, 1996); however splenectomy may be necessary (Galindo et al., 1999). Low dose aspirin and subcutaneous unfractionated heparin has proven to be effective in preventing recurrent fetal loss (Rai et al., 1997; Kutteh, 1996; Kutteh and Ermel, 1996).
Table 1. Preliminary Classification Criteria for Definite Antiphospholipid Syndrome (Wilson et al., 1999).

### Clinical Criteria

1. **Vascular thrombosis:**
   
   One or more episodes of arterial, venous, or small vessel thrombosis, in any tissue or organ. Thrombosis must be confirmed by imaging or Doppler studies or histopathology, with the exception of superficial venous thrombosis. For histopathologic confirmation, thrombosis should be present without significant evidence of inflammation in the vessel wall.

2. **Pregnancy morbidity**
   
   (a) One or more unexplained deaths of a morphologically normal fetus at or after the 10th week of gestation, with fetal morphology documented by ultrasound or by direct examination of the fetus, or
   
   (b) One or more premature births of a morphologically normal neonate at or before the 34th week of gestation because of severe preeclampsia or eclampsia, or severe placental insufficiency, or
   
   (c) Three or more unexplained consecutive spontaneous abortions before the 10th week of gestation, with maternal anatomic or hormonal abnormalities and paternal and maternal chromosomal causes excluded.

### Laboratory Criteria

1. Anticardiolipin antibody of IgG and/or IgM isotype in blood, present in medium or high titer, on 2 or more occasions, at least 6 weeks apart, measured by a standardized ELISA for β2-glycoprotein I-dependent anticardiolipin antibody.

2. Lupus anticoagulant present in plasma, on 2 or more occasions at least 6 weeks apart, detected according to the guidelines of the International Society of Thrombosis and Haemostasis (Scientific Subcommittee on Lupus Anticoagulants/Phospholipid-Dependent Antibodies), in the following steps:
   
   (a) Prolonged phospholipid-dependent coagulation screening test (e.g. activated partial thromboplastin time), kaolin clotting time, dilute Russell's viper venom time, dilute prothrombin time, and Textarin time.
   
   (b) Failure to correct the prolonged coagulation time by mixing with normal platelet-poor plasma.
   
   (c) Shortening or correction of the prolonged coagulation time on the screening test by the addition of excess phospholipid.
   
   (d) Exclusion of other coagulopathies as clinically indicated e.g. factor VIII inhibitor and heparin, as appropriate.

Definite antiphospholipid antibody syndrome is considered to be present if at least 1 of the clinical criteria and 1 of the laboratory criteria are met.
1.3 β2-Glycoprotein I

1.3.1 Characterization of β2GPI

Human β2-glycoprotein I (β2GPI) is also known as apolipoprotein H (Apo H). β2GPI is a 54 kDa glycoprotein with a plasma concentration of 200 μg/mL that binds to negatively charged phospholipid (Wurm et al., 1982). It was first purified by Schulze et al. in 1961 (Schulze et al., 1961). The amino acid sequence of β2GPI has been determined by amino acid sequence analysis (Lozier et al., 1984) and cDNA cloning (Mehdi et al., 1991; Matsuura et al., 1991; Kristensen et al., 1991; Steinkasserer et al., 1991) with differences reported at 2 amino acids. Amino acid sequencing showed Cys102 and Asn169 in the sequence while all cDNA cloning experiments showed Ser102 and Cys169. The gene structure encoding for β2GPI has also been determined (Okkels et al., 1999). The mature β2GPI protein is composed of 326 amino acids arranged in five short consensus repeats (SCRs) which are also known as Sushi-domain and complement control protein modules (Figure 1). SCRs are approximately 60 amino acids in length and contain four conserved cysteine residues which form disulfide bridges in Cys 1-3 and Cys 2-4 fashion (Reid et al., 1986). Numerous proteins belonging to the Regulation of Complement Activation (RCA) gene cluster also consist of multiple SCRs (Rodriguez et al., 1985). Despite the structural similarity, β2GPI does not map to the same locus as the RCA gene cluster. cDNA cloning studies mapped the structural locus for this protein to q23 → qter on chromosome 17 and not part of the RCA cluster on chromosome 1 (Nukatsuka and Nagasawa, 1987).
Figure 1. Primary structure of human β2GPI.

β2GPI is composed of five domains. "CHO" in domain III and domain IV represents the N-glycosylation sites. This schematic diagram is as shown by Koike, T. with modifications (Koike, 1994).
1.3.2 Carbohydrate Composition and Function of Sugar Residues

β2GPI has four N-glycosylation sites. They are located at Asn143, Asn164, Asn174 of domain III, and Asn234 of domain IV (Mehdi et al., 1991; Steinkasserer et al., 1991). Carbohydrate analyses of β2GPI show that it is rich in carbohydrates including mannose, N-acetylglucosamine, sialic acid, N-acetylgalactosamine, galactose, and fucose (Gambino et al., 1997; Gambino et al., 1997; Gambino et al., 1997). These carbohydrate residues have been suggested to contribute to phospholipid binding (Brighton et al., 1999) and to the integrity of the secondary structures of β2GPI (Walsh et al., 1990) but not in the recognition of β2GPI by aPL (Arvieux et al., 1994). O-linked carbohydrates are also present; however, the O-glycosylation sites remain to be determined (Gambino et al., 1997).

1.3.3 Binding of β2GPI to Phospholipids

β2GPI can interact with anionic phospholipids such as cardiolipin (CL), phosphatidylserine (PS), phosphatidylinositol (PI), and phosphatidic acid (PA) (Wurm, 1984). Interaction with phosphatidylethanolamine has also been reported (Harper et al., 1998). A cluster of lysine residues in the fifth domain of β2GPI has been identified to mediate phospholipid binding (Kertesz et al., 1995). These lysines reside in the C281KNKEKKC288 sequence. Site-directed mutagenesis analysis showed that Lys284, Lys286, and Lys287 are critical for anionic phospholipid binding (Sheng et al., 1996). These lysine residues are also essential for β2GPI binding to endothelial cells (Del Papa et al., 1997).
et al., 1998). A study of bovine β2GPI also showed that the besides the fifth domain, the first domain may also be involved in phospholipid binding (Hagihara et al., 1995).

Another region of β2GP1 has been suggested to mediate phospholipid binding. This region corresponds to amino acid K^{268}NGMLHGDKVS^{278} of the fifth domain with His273 appearing to be indispensable for the binding to CL. Substitutions with other amino acids in this position decrease the binding ability of β2GP1 with anionic phospholipids (Lauer et al., 1993).

1.3.4 β2GPI Polymorphism and Mutation

Four alleles of β2GPI, APO H*1 TO APO H*4, have been identified by isoelectric focusing (Kamboh et al., 1988). APO H*3 can be further characterized into two subtypes; APO H*3^w and APO H*3^b, based upon its reactivity with monoclonal antibody 3D11. APO H*2 is the most prevalent allele in North America. APO H*1 has a S88N mutation whereas APO H*3^w has a W316S (Sanghera et al., 1997).

Phospholipid binding studies showed that β2GPI with C306G and/or W316S mutation(s) cannot bind anionic phospholipid. The W316S mutation disrupts the conformation of four highly conserved hydrophobic amino acids sequence at positions 313-316, which is a potential protein-lipid hydrophobic interaction site (Sanghera et al., 1997). However, the β2GPI W316S mutation does not confer protection against anti-β2GPI autoantibody production even though β2GPI cannot bind phospholipid for anti-β2GPI binding (Horbach et al., 1998). The C306G mutation causes the disruption of a disulfide bond between Cys281 and Cys306 and affects the conformation of positively charged lysine residues along with four hydrophobic amino acids sequence (Sanghera et
Polymorphic substitution at Val247 by Leu247 has been reported but appeared not to correlate with the four isoelectric focusing alleles (Steinkasserer et al., 1993).

1.3.5 Physical Studies of β2GPI

An intrinsic fluorescence study measuring the blue shift (345 to 334 nm) of β2GPI upon binding phosphatidylglycerol (PG)-containing phospholipid membrane showed that tryptophan residues of β2GPI move from a polar to a nonpolar environment. This finding suggests that β2GPI binding to phospholipid involves insertion of part of the protein into the PG-containing membrane (Wang et al., 1999). This hydrophobic interaction between β2GPI and phospholipid as part of the β2GPI-phospholipid binding process has also been shown using phospholipid monolayer technique and membrane-insertion analysis (Wang et al., 1998). The crystal structure of β2GPI indicates a region of 14 cationic amino acid residues for electrostatic interactions with anionic phospholipid headgroups and an exposed membrane-insertion loop for phospholipid binding (Bouma et al., 1999). Using infrared spectroscopy, changes in β2GPI structure upon binding to cardiolipin was detected (Borchman et al., 1995). Free β2GPI was calculated to contain 18% turns, 37% alpha-helix, and 45% beta-sheet structure. After binding to cardiolipin, there was a decrease in beta-sheet structure of β2GPI from 46% to 23% and appearance of 26% to 28% random structure.
1.3.6 Interactions of β2GPI with Different Cells, Proteins, and Biochemicals

β2GPI has affinity towards a variety of targets including activated platelets
(Vazquez-Mellado et al., 1994; Nimpf et al., 1985; Schousboe, 1983), endothelial cells
(George et al., 1998; Del Papa et al., 1998; Del Papa et al., 1997; Del Papa et al., 1997; Del Papa et al., 1995; Le Tonqueze et al., 1995), lipoproteins (Cardigan et al., 1998; McNally et al., 1996; Polz et al., 1981; Polz et al., 1980), anionic liposomes
(Balasubramanian and Schroit, 1998; Balasubramanian et al., 1997), as well as apoptotic bodies (Levine et al., 1998; Rovere et al., 1998; Manfredi et al., 1998; Price et al., 1996; Manfredi et al., ). β2GPI binds to Staphylococcus aureus leading to speculation that aPL production may be linked to S. aureus infection (Zhang et al., 1999). β2GPI also interacts with calmodulin in kidney, however, the physiological significance remains to be determined (Røjkjær et al., 1997; Klærke et al., 1997). Other biological substances that β2GPI binds include heparin (Polz et al., 1980) and DNA (Kroll et al., 1976).

1.3.7 Interaction Among β2GPI, Platelet, Coagulation, and Anticoagulation

Numerous effects of β2GPI on platelets and hemostasis have been demonstrated. These studies suggest that β2GPI has coagulation inhibition properties. β2GPI inhibits factor X activation (Ieko et al., 1998; Shi et al., 1993) and prothrombinase activity of activated platelets (Nimpf et al., 1986) as well as platelet serotonin release during ADP-dependent platelet aggregation (Nimpf et al., 1987). Increase in adenylate cyclase activity by β2GPI in platelet membrane has been also reported (Schousboe, 1983). β2GPI can inhibit contact activation of the intrinsic pathway (Schousboe, 1988; Schousboe and Rasmussen, 1988; Schousboe, 1985) as well as prekallikrein activation.
In addition, β2GPI can inhibit factor XII activation by lipoprotein lipase on very low density lipoproteins (McNally et al., 1996).

In the anticoagulation mechanism, β2GPI may be an inhibitor of activated protein C (APC) (Canfield and Kisiel, 1982). Using clotting assays, β2GPI inhibits APC and produces a procoagulant condition (Mori et al., 1996). These studies suggest a procoagulant role for β2GPI. In contrast, the observation of β2GPI mediated liberation of free protein S from protein S-C4b binding protein complex, an important anticoagulation cofactor, suggests an anticoagulant role for β2GPI in anticoagulation (Merrill et al., 1999; Atsumi et al., 1997). Also, it has been suggested that it may be a natural anticoagulant (Brighton et al., 1996). In this study, β2GPI levels along with other anticoagulant proteins were reduced in patients with disseminated intravascular coagulation (DIC). This suggests specific consumption of β2GPI in in vivo coagulation. Therefore, the precise role(s) of β2GPI in coagulation remains unclear. It is possible that it functions as both procoagulant and anticoagulant. However, this remains to be determined.

1.3.8 β2GPI and Fibrinolysis

β2GPI contains a cleavage site for plasmin between the residues Lys317 and Thr318 in the fifth domain (Horbach et al., 1999; Ohkura et al., 1998). The cleavage leads to significant reduction in β2GPI affinity to anionic phospholipid (Horbach et al., 1999; Ohkura et al., 1998; Hagihara et al., 1997). In plasma of patients with DIC, the increase in cleaved β2GPI level is accompanied by a decrease in the level of intact β2GPI, suggesting active consumption of β2GPI by plasmin during DIC (Horbach et al., 1999).
Lipoprotein (a) (Lp(a)) is structurally similar to plasminogen (McLean et al., 1987). Lp(a) impairs fibrinolytic activity by competing with plasminogen and tissue type-plasminogen activator for fibrin-binding sites (Loscalzo et al., 1990). Lp(a) interaction with B2GPI has been reported (Kochl et al., 1997). Elevated Lp(a) (Yamazaki et al., 1994) with concurrent depressed fibrinolytic activity as indicated by lower D-dimer and higher plasminogen activator inhibitor in APS patients has been observed (Atsumi et al., 1998). Correlations among Lp(a) levels, B2GPI immune complex levels, and the occurrence of thrombocytopenia are also found in APS patients (George et al., 1999). B2GPI interactions with plasmin and Lp(a) together suggest that B2GPI may be involved in fibrinolysis.

1.3.9 B2GPI and Lipoproteins

B2GPI binds all major lipoprotein density fractions, especially chylomicrons, very low density lipoprotein (VLDL), and high density lipoprotein (HDL) (Polz and Kostner, 1979) and activates lipoprotein lipase (Nakaya et al., 1980). B2GPI may be involved in the removal of triglyceride from circulating blood (Wurm et al., 1982). The level of B2GPI is elevated significantly in patients with hypercholesterolaemia, hypertriglyceridaemia, mixed hyperlipidaemia (McNally et al., 1994), non-insulin dependent diabetes mellitus and insulin-dependent diabetes mellitus (Cassader et al., 1997). Individuals with the APO H*3 allele of B2GPI have significantly higher triglyceride level (Cassader et al., 1994). Despite these observations of B2GPI associations with lipoprotein, patients with B2GPI deficiency do not show signs of significant perturbation of normal lipoprotein metabolism (Hoeg et al., 1985).
β2GPI inhibits *in vitro* uptake of oxidized low density lipoprotein (LDL) by macrophages and the presence of anti-β2GPI can reverse the process as well as accelerate the uptake of oxidized LDL by macrophages. These observations suggest an antiatherogenic function for β2GPI that could be abrogated by anti-β2GPI (Hasunuma *et al*., 1997). With regards to oxidized lipid, anti-β2GPI antibodies have increased affinity for β2GPI coupled to oxidized cardiolipin (Horkko *et al*., 1997). In addition, significantly higher level of lipid peroxidation has been measured in APS patients (Pratico *et al*., 1999). These studies together suggest that oxidation of lipids enhance β2GPI-mediated anti-β2GPI binding to lipoproteins or cell membranes.

Anti-β2GPI antibodies induce accelerated atherosclerosis in a LDL-receptor-deficient mouse model fed on a high-cholesterol diet (George *et al*., 1999; George *et al*., 1998) as well as in an apolipoprotein E-knockout mouse model (George *et al*., 1999). This suggests that anti-β2GPI may inhibit the putative anti-atherogenic function of β2GPI.

### 1.3.10 β2GPI and Apoptosis

Several studies have reported that β2GPI is involved in apoptosis. β2GPI and anti-β2GPI deposit on apoptotic thymocytes (Price *et al*., 1996). The formation of aPL is induced in mice after immunization with heterologous β2GPI bound to apoptotic thymocytes (Levine *et al*., 1998). Also, β2GPI can mediate anti-β2GPI binding to apoptotic cells for macrophage (Manfredi *et al*., 1998; Manfredi *et al*.,) and dendritic cell recognition (Rovere *et al*., 1998). The observations of β2GPI-mediated clearance of

18
anionic liposomes by macrophages (Balasubramanian and Schroit, 1998; Balasubramanian et al., 1997) further suggest β2GPI is involved in the apoptotic process.

1.3.11 Anti-β2GPI Binding to β2GPI

Binding studies have shown that patients' anti-β2GPI binds to the first domain (Iverson et al., 1998), the fourth domain (George et al., 1998), as well as the fifth domain of β2GPI (Yang et al., 1998; Wang et al., 1995). Monoclonal antibodies from mice immunized with human β2GPI bind β2GPI in the first to fourth domains (Igarashi et al., 1996). The reason for the difference in binding observed in these studies awaits further investigation.

1.4 Anticoagulation

1.4.1 Coagulation

The coagulation system is activated in response to vascular damage (Figure 2). The initial step involves the exposure of tissue factor at the injured site (Drake et al., 1989) for factor VIIa binding (Nemerson, 1988). The association of these two proteins leads to the activation of factor IX and factor X to become factor IXa and Xa (Österud and Rapaport, 1977) which bind anionic phospholipid surfaces such as activated platelets (DiScipio et al., 1977). Factor Xa then activates prothrombin to thrombin (Mann et al., 1988). With the generation of thrombin, soluble fibrinogen molecules are converted into an insoluble fibrin mesh at the injured site to hinder bleeding (Blombäck and Blombäck, 1986).
At the same time, thrombin activates factor VIII (Hill-Eubanks et al., 1989) and factor V (Kane and Davie, 1988). The resulting factor VIIIa and factor Va bind to anionic phospholipid, and form complexes with factor IXa and factor Xa, respectively. The VIIIa-IXa and Va-Xa complexes are known as tenase and prothrombinase and are potent enzymatic activators for factor X (Østerud and Rapaport, 1970; Lundblad and Davie, 1964) and prothrombin (Mann et al., 1988), respectively. The generation of these two complexes leads to an amplification of thrombin generation for further fibrin production to arrest blood loss. Therefore, the VIIIa- and Va-containing tenase and prothrombinase complex play a central role in the amplification of thrombin generation and fibrin deposition at the site of injury.

The coagulation activities of tenase and prothrombinase are regulated by the anticoagulation mechanism to prevent uncontrolled promotion of blood clotting. Activated protein C (APC) and protein S are the two proteins with anticoagulant activity involved in the regulation by proteolytically cleaving and inactivating Va (Kalafatis et al., 1994) and VIIIa (Fay et al., 1993; Fay et al., 1991) of prothrombinase and tenase.
Figure 2. Scheme of Tissue Factor Pathway of Coagulation and Activated Protein C Anticoagulation System.

Thick arrows represent inactivation of factor VIIIa and Va by APC.

**Tissue Factor (TF)**

- VIIa
  - TF-VIIa
    - IX → IXa
    - VIII → VIIIa
      - VIIIaIXa (Tenase)
        - X → Xa
          - V → Va
            - VaXa (Prothrombinase)
              - Prothrombin → Thrombin
                - Thrombomodulin (TM)
                  - TM-Thrombin
                    - Protein C → APC
1.4.2 Activated Protein C

1.4.2.1 Characterization of Activated Protein C

APC is generated by the proteolytic activity of thrombin-thrombomodulin complex on its precursor protein C (Esmon and Owen, 1981). Protein C is a 62 kDa protein (Kisiel, 1979; Kisiel, 1979) with a plasma concentration of 4 µg/mL (64 nM) (Griffin et al., 1982). It belongs to the vitamin K-dependent protein family whose members contain gamma-carboxyglutamic acid (Gla). Other proteins that belong to this family are blood clotting factor II, VII, IX, and X, as well as the APC cofactor protein S (Mann et al., 1988). Protein C contains multiple domains. The domains starting from the N-terminus are the Gla-domain, two epidermal growth factor (EGF)-like domain, and a serine protease domain (Beckmann et al., 1985; Foster and Davie, 1984). It is synthesized in the liver as a single polypeptide chain of 461 amino acids (Beckmann et al., 1985; Foster and Davie, 1984). Lys156 and Arg157 are removed from the serine protease domain of the polypeptide resulting in a mature protein C with one heavy chain and one light chain linked together through an inter-chain disulfide bridge (Foster et al., 1991).

The activation of protein C to APC requires the presence of thrombin and an integral membrane protein on endothelial cells known as thrombomodulin (TM) (Esmon and Owen, 1981). As the coagulation reaction progresses during blood clotting with an increasing amount of thrombin formation, thrombin provides negative feedback to terminate the reaction. Thrombin inhibits coagulation by forming a complex with TM. TM-bound thrombin loses its procoagulant activity and acquires potent protein C
activation activity (Esmon et al., 1983; Esmon et al., 1982). As a result of thrombin binding to TM, APC is generated from protein C. The thrombin-TM complex activates protein C by cleaving between Arg169 and Leu170 (Kisiel, 1979).

1.4.2.2 APC Catalyzed FVa and FVIIIa Inactivation

The major reactions the APC catalyzes are the inactivation of FVa and FVIIIa. FVa and FVIIIa are homologous proteins (Kane and Davie, 1988). FVa has three cleavage sites for APC, namely Arg306, Arg506, and Arg679. Cleavage at Arg506 is required for efficient cleavage of Arg306 and Arg679. Arg306 is the major inactivating cleavage site resulting in the loss of most FVa activity and cleavage of Arg679 accounts for the remaining FVa activity (Kalafatis et al., 1994). FVIIIa has two cleavage sites for APC at Arg336 and Arg562. Cleavage at Arg336 by APC leads to the dissociation of FVIIIa (Fay et al., 1993), whereas the cleavage at Arg562 removes the interactive site with FIXa (Fay et al., 1991).

1.4.3 Protein S

1.4.3.1 Characterization of Protein S

Human protein S is a 75 kDa protein synthesized as a single polypeptide chain of 635 amino acids (DiScipio and Davie, 1979) in hepatocytes, endothelial cells, megakaryocytes. It has a plasma concentration of 20 μg/mL, 290nM (Schwarz et al., 1986; Fair and Revak, 1984; Dahlback, 1983b). Protein S is composed of multiple discrete domains (Stenflo et al., 1987; Dahlback, 1986). The domains starting from the N-terminus are the vitamin K-dependent gamma-carboxyglutamic acid (Gla) rich region
and followed by a thrombin-sensitive region and four EGF-like domains (Doolittle et al., 1984), and a region homologous to sex hormone binding globulin (SHBG) (Baker et al., 1987). The thrombin-sensitive region and the first EGF-like domains are thought to be involved in protein S and activated protein C interactions (Dahlback et al., 1990). Studies have suggested that amino acids 420 - 434 in the SHBG-like domain are important for the interaction with C4BP (Greengard et al., 1995).

In plasma, approximately 60% of the protein S is present as a 1:1 stoichiometric complex with C4b-binding protein (C4BP) with β-chain, and the remaining is present as free protein (Dahlback and Stenflo, 1981). The interaction between the protein S and C4BP is non-covalent; the C4BP-bound protein S loses its cofactor activity for APC. In the absence of calcium, C4BP-protein S complex has a $K_d$ of $1 \times 10^{-7}$ M (Dahlback and Stenflo, 1981), whereas in the presence of calcium, $K_d$ is $5 \times 10^{-10}$ M (Dahlback, 1983a). Calcium, therefore, promotes their interaction dramatically. The low $K_d$ in the presence of calcium suggests that in vivo almost all C4BP with β-chain is bound to protein S, thus free protein S levels are determined by the level of β-chain containing C4BP.

1.4.3.2 Protein S as Cofactor of Activated Protein C

With the activation of the anticoagulation process, APC forms a 1:1 stoichiometric complex with protein S on anionic phospholipid vesicles (Walker, 1981) and catalyzes the proteolytic inactivation of phospholipid-bound factor Va and factor VIIIa (Marlar et al., 1981). Protein S increases APC activity by relocating the active site of APC about 1 nm closer to the membrane surface (Yegneswaran et al., 1999; Yegneswaran et al., 1997). This effect is mediated by the interaction between the
thrombin-sensitive region and the first EGF-like domain in protein S (Dahlback et al., 1990) with the Gla-domain and/or the first EGF-like domain of APC (Ohlin et al., 1990). Arg49 and Gln52 in the thrombin-sensitive region and Lys97 and Pro106 in the first EGF domain of protein S appear to be crucial for APC cofactor activity (He et al., 1998).

1.4.4 Anticoagulation and Antiphospholipid Syndrome

The anticoagulation activity of activated protein C (APC) is impaired by the presence of antiphospholipid antibodies aPL. Several studies have demonstrated that aPL can inhibit FVa inactivation by APC (Galli et al., 1998; Oosting et al., 1993; Malia et al., 1990; Marciniak and Romond, 1989). In another study, 47% of 78 aPL in patient samples bound protein C via β2GPI in the presence of phospholipid (Atsumi et al., 1998). Therefore, inhibition of APC activity is a prevalent feature for APS patients. Clinical data from patients with primary or secondary antiphospholipid syndrome report a variable decrease in the immunological level of plasma free protein S (Ames et al., 1996; Ginsberg et al., 1995). It has also been suggested that protein S-C4BP complex formation can be down-regulated by β2GPI and this down-regulation can be disrupted by anti-β2GPI antibodies (Merrill et al., 1999; Atsumi et al., 1997).
1.5 Complement

1.5.1 Complement System

The complement system has an important role in innate immune defense. The major biologic functions of complement include cytolysis, inflammation, immune complex clearance, and particle opsonization. The complement system consists of at least 25 different proteins that are involved in the activation and regulation of the enzyme cascade. The system is divided into 2 major pathways known as the classical pathway and alternative pathway. Activation of either pathway results in the formation of the cytolytic C5b-9 complex, which is also known as the membrane attack complex (MAC). MACs form pores in the membrane of cells through which ions and small molecules can pass into the cell, cause cell swelling, and eventually cell lysis. The classical pathway can also be activated by mannose-binding lectin (MBL) (Matsuhita and Fujita, 1992). This is known as the lectin pathway. C-reactive protein (CRP) (Osmand et al., 1977) and serum amyloid P (SAP) (Bristow and Boackle, 1986; Pepys and Baltz, 1983; Pepys et al., 1982) that constitute the pentraxin pathway are another two proteins that activate the classical pathway (Figure 3).
Figure 3. The Complement System.

Pentraxin Classical Pathway
CRP/SAP Immunglobulins

Lectin Pathway
MBL

Alternative Pathway

C1
Cl
C4 C4b C4a C2
C4b2 C4b2a C3
C3
C3a C3b C3bBb C3bBb3b C5 C5a C5b C5b-9
C3b C3bBb C3(H2O)B C3(H2O)Bb C3a
C6 C7 C8 C9

Membrane Attack Complex (MAC)
1.5.1.1 Classical Pathway

Activation of the classical pathway involves the sequential activation of C1, C4, C2, C3, and C5. Activation of the multi-subunit C1 complex, which contains one Clq, twoClr, and twoCls, can be initiated by IgM, and IgG (Sim and Reid, 1991). In humans, IgG3 and IgG1 subclasses are strong complement activators, IgG2 is a weak activator, and IgG4 is generally considered not to activate complement (Hamilton, 1987). C1 becomes activated by binding to a membrane surface or a particle coated with immunoglobulin. Activated C1 then cleaves C4, releasing C4a and C4b fragment (Schreiber and Muller-Eberhard, 1974). After the cleavage, C4b fragment binds covalently to the membrane surface close to activated C1. C2 then binds to C4b deposited on the membrane surface and is cleaved by the activated C1 nearby. After the cleavage, C2b is released with C2a remaining bound to C4b as a C4b2a complex, which is also referred to as C3 convertase (Nagasawa and Stroud, 1977). This C3 convertase then converts C3 into C3a and C3b fragments with C3b bound covalently to the membrane close to C4b2a, forming C5 convertase. C5 then binds to the C3b portion of the C5 convertase on the membrane and is cleaved by the C2a portion of the C5 convertase (Vogt et al., 1978). As a result of the cleavage, C5b is generated and remained bound to C3b, releasing C5a. The formation of C5b initiates the formation of a lytic pore on cell surface by allowing the assembly of the remaining components of the membrane attack complex, namely, C6 (Lachmann and Thompson, 1970), C7 (DiScipio et al., 1988), C8 (Kolb and Müller-Eberhard, 1976), and C9 (Podack and Tschopp, 1982).
1.5.1.2 Alternative Pathway

The spontaneous activation of C3 by a water molecule to form C3(H₂O) is the first step of alternative pathway activation (Lachmann and Hughes-Jones, 1984). C3(H₂O) interacts with factor B in fluid phase. Once factor B is associated with C3(H₂O), it is cleaved by factor D to become a C3(H₂O)Bb complex with fragment Ba released (Fearon et al., 1973). C3(H₂O)Bb is a fluid phase C3 convertase that cleaves C3 to promote the deposition of C3b to membrane surface. If the C3b generated is deposited onto an activating surface such as microbial pathogens or surfaces that promote the binding of factor B, factor B then associates with the membrane bound C3b. Factor D then cleaves membrane-bound factor B to become a surface bound C3bBb complex (Fearon et al., 1973). This complex is analogous to the C4b2a complex in that they are both C3 convertases that cleave C3. C3bBb cleaves C3, releasing C3a, and becomes C3bBb3b, a C5 convertase (Daha et al., 1976). C5 then binds to the C3b portion of the C5 convertase on the membrane and is cleaved by the Bb portion of the C5 convertase. As a result of the cleavage, C5b is generated and remains bound to C3b, releasing C5a (Schreiber et al., 1978). Similar to the classical pathway, the formation of C5b can initiate the formation of a lytic pore on cell surface by allowing the assembly of the remaining components of the membrane attack complex, namely, C6, C7 C8, and C9.

Despite the constant activation of C3 to C3(H₂O), alternative pathway activation does not occur on host cell surface. This is because C3(H₂O) is only generated at a very low level. Also, the host cell is a poor surface for supporting alternative pathway
activation because host cells do not favor factor B binding. Also, the presence of complement regulators would quickly degrade any C3b deposited on the host cell.

1.5.2 Complement Regulation

Complement is a cascade system with its action amplified as the cascade proceeds. Also, the complement system does not distinguish between host cells and invader cells. Therefore, a stringent control mechanism is required to prevent uncontrolled complement activation as well as lysis of host cells. Complement activation is tightly controlled by specific regulatory proteins that act on different complement proteins in the classical pathway, the alternative pathway, and the common pathway. These complement regulators may be either plasma proteins or membrane bound protein on host cells (Figure 4). Plasma inhibitors include C1-inhibitor (C1-inh) (Sim et al., 1979; Ruddy and Austen, 1969), C4b-binding protein (C4BP) (Fujita and Nussenzweig, 1979; Fujita et al., 1978; Scharffstein et al., 1978), factor H (Whaley and Ruddy, 1976), factor I (Fujita and Nussenzweig, 1979), vitronectin (S-protein) (Podack et al., 1984), and clusterin (SP-40,40) (Jenne and Tschopp, 1992). Membrane bound inhibitors include decay-accelerating factor (DAF, CD55) (Nicholson-Weller et al., 1982), membrane cofactor protein (MCP, CD46) (Cole et al., 1982), complement receptor 1 (CR1, CD35) (Wong, 1990), CD59 (membrane inhibitor of reactive lysis; MIRL) (Davies et al., 1989), and homologous restriction factor (HRF, C8 binding protein) (Zalman et al., 1986).
Figure 4. Regulation in the Complement System. These complement regulators exist as plasma protein as well as membrane bound protein on host cells. Plasma inhibitors include C1-inhibitor (C1-inh), C4b-binding protein (C4BP), factor H, factor I, vitronectin (S-protein), and clusterin (SP-40,40). Membrane bound inhibitors include decay-accelerating factor (DAF, CD55), membrane cofactor protein (MCP), complement receptor 1 (CR1), CD59 (membrane inhibitor of reactive lysis; MIRL), and homologous restriction factor (HRF). Thick arrows represent action of complement regulatory proteins.

**Classical Pathway**

C1 \(\rightarrow\) C4b \(\rightarrow\) C4b2 \(\rightarrow\) C4b2a3b \(\rightarrow\) C4BP Factor I 

**Alternative Pathway**

C3 \(\rightarrow\) C4b \(\rightarrow\) C3b \(\rightarrow\) Factor B \(\rightarrow\) Factor D \(\rightarrow\) C3bBb \(\rightarrow\) Factor H Factor I 

Factor H 

C5 \(\rightarrow\) C5b \(\rightarrow\) C5b6 \(\rightarrow\) C5b-7 \(\rightarrow\) C5b-8 \(\rightarrow\) C5b-9 

S protein (vitronectin) Clusterin (SP-40,40) 

CD59 (MIRL) HRF (C8BP)
1.5.3 C4b-Binding Protein

1.5.3.1 Characterization of C4b-Binding Protein

C4BP is an oligomeric glycoprotein that regulates the activation of the classical pathway of the complement system (Fujita and Nussenzweig, 1979; Fujita et al., 1978; Scharfstein et al., 1978). C4BP is present in plasma at a concentration of 250 µg/mL (500 nM) with a characteristic molecular heterogeneity. Isoforms of C4BP exist differing by the number of α-chains and presence of the β-chain. About 80% of C4BP has a molecular weight of 590 kDa and is composed of seven α-chains and a single protein S-binding β-chain joined together by interchain disulfide bridges in the C-terminal region (Hillarp and Dahlback, 1988; Dahlback, 1983a; Reid and Gagnon, 1982; Scharfstein et al., 1978). The remaining C4BP is mostly an isoform containing only seven α-chains that exists as free C4BP with a molecular weight of 540 kDa. Alternative forms of C4BP also include heptamer of six α-chains and one β-chain (Dahlback, 1983a).

The amino acid sequence of the α-chains of C4BP has been determined by a combination of cDNA cloning and peptide sequencing (Lintin and Reid, 1986; Chung et al., 1985). Each α-chain has 549 amino acid residues. The N-terminal 491 residues contain eight homologous SCRs. The C-terminal 58 residues appear to form a stable α-chain containing two cysteine residues for interchain disulfide linkage (Chung et al., 1985). The amino acid sequence of the β-chain has also been determined by cDNA cloning (Chung et al., 1985) and peptide sequencing (Chung et al., 1985). The β-chain has 235 amino acids as well as five potential glycosylation sites. However, actual sites
that are glycosylated have not been identified. The N-terminus of the β-chain can be divided into three SCRs, therefore it is very similar to the α-chain, although the length is shorter. Furthermore, the C-terminal region of the β-chain is also very similar to the corresponding region of the α-chain. The C-terminal region of both chains contains two cysteine residues that are involved in the interchain disulfide bonds of the molecule.

1.5.3.2 Mechanism of Complement Inhibition by C4BP

C4b-binding protein (C4BP) is a classical pathway activation inhibitor. C4BP modulates the activation by accelerating the dissociation of C4b2a by competing with C2a for the binding site on C4b (Gigli et al., 1979) as well as functioning as a cofactor for factor I in the proteolytic degradation of C4b (Nagasawa et al., 1980; Fujita and Nussenzweig, 1979; Fujita et al., 1978). The α-chain contains the binding site for complement component C4b and contains factor I cofactor activity (Fujita et al., 1978), (Fujita and Nussenzweig, 1979). Under physiological conditions, each C4BP molecule binds around 4.8 C4b (Kd 8.3 x 10^-8 M) molecules even though it has seven C4b binding sites (Ziccardi et al., 1984). Studies using proteolytic fragments of C4BP α-chain generated by chymotrypsin, trypsin, and pepsin indicated that the factor I cofactor activity is located in residues 177 – 322 (Chung and Reid, 1985). The binding sites for C4b reported by different groups have been contradictory. Using proteolytic fragment of α-chain generated by chymotrypsin or trypsin, the C4b binding activity may be located in residues 332-395, corresponding to the sixth and seventh SCRs (Chung et al., 1985). Using monoclonal antibody that inhibits C4b binding to α-chain, the epitope of the monoclonal antibody was mapped to a region between Ser333 and Arg356 in SCR6.
(Hessing et al., 1993). Both of these studies suggest C4b-binding site to be in the carboxyl terminal half of the α-chain. In contrast, an electron microscopic study of C4BP-C4b complex suggested a site closer to the amino terminal (Dahlback et al., 1983). Also, chimeric α-chains with one, two, or three SCRs replaced by the corresponding SCRs in the β-chain did not support C4b binding and suggested the C4b binding site to be near the amino terminal of α-chain. In addition, monoclonal antibodies against SCR1 and SCR2 inhibited C4b binding (Hardig et al., 1997). Murine C4BP is homologous to human C4BP (51% identity) except for SCRs corresponding to SCR5 and SCR6 in human are not present. Without these SCRs, murine C4BP still has the ability to bind human C4b (Kristensen et al., 1987). This strongly suggests that the amino terminal is the more relevant region for C4b binding (Morgan and Harris, 1999).

1.5.3.3 C4BP Interaction with Protein S

The β-chain also contains the binding site for the anticoagulation protein, protein S (Dahlback, 1983b). Protein S and C4BP form a 1:1 non-covalent complex (Dahlback, 1984). The residues involved in the complex formation are residues 31 - 45 (Fernandez and Griffin, 1994). Approximately 60% of protein S is complexed with C4BP, and complex formation eliminates protein S cofactor activity as an anticoagulant. However, complex formation does not interfere with C4BP activity as an inhibitor of complement system (Dahlback et al., 1990; Dahlback and Stenflo, 1981). The physiological significance of protein S binding to the C4BP β-chain in complement activation and anticoagulation is unclear to date. It has been suggested that protein S may be involved in complement regulation by localizing C4BP on anionic phospholipid surfaces, such as
activated platelet surface, for the control of local classical pathway complement
activation (Dahlback, 1984). Recently, β2GPI has been shown to inhibit protein S-C4BP
binding and to liberate free protein S from the C4BP-protein S complex (Merrill et al.,
1999). Protein S bound to C4BP is resistant to chymotrypsin, leukocyte elatase and
cathepsin G. This could suggest that binding of C4BP to protein S protects protein S
from proteolytic degradation at the site of inflammation during inflammatory responses
by an increased C4BP levels during this period (Steinkasserer et al., 1992). A recent
report suggested the hypothesis that control of the protein S system is aimed at
maintaining normal free protein S level during inflammatory response by an increase in
the total protein S level (Carr and Zekert, 1993). During inflammatory response, the
level of C4BP increases. The increase in C4BP is counteracted by an increase in total
protein S. Thus, normal free protein S level and anticoagulation cofactor activity are both
maintained.

1.5.4 MBL

1.5.4.1 Characterization of MBL

MBL lectin is also known as mannose binding protein, mannan binding lectin,
mannan binding protein, core-specific lectin, and Ra-reactive factor. It is an oligomeric
protein made up of two to six subunits with a serum concentration of 1780 ng/mL (3.7
nM) (Lau et al., 1995). Each of these subunits (96 kDa) is composed of three identical
polypeptides (32 kDa). Starting from the N-terminus, the polypeptide chain is divided
into four domains, namely, the cysteine-rich domain, the collagen-like domain, the ‘neck’
region, and the carbohydrate-recognition domain. The collagen-like domain is a region containing 18-20 repeats of Gly-Xaa-Yaa. The three polypeptide chains assemble into a subunit by forming the collagen triple helix connected to the carbohydrate-recognition domain through the neck region. The structure of the subunit is stabilized by the interchain disulfide bonding in the cysteine-rich domain. These subunits further interact to assemble an oligomeric structure by inter-subunit disulfide bridges and/or non-covalent interactions (Turner, 1996).

MBL belongs to a family of proteins known as collectins, a group of collagen-containing mammalian lectins. In human, the MBL gene is encoded on the long arm of chromosome 10 (Kolble and Reid, 1993; Sastry et al., 1989) with 4 exons corresponding to the four domains of the protein (Taylor et al., 1989; Ezekowitz et al., 1988). This family includes three human lung surfactant proteins SP-D, SP-A1, and SP-A2, bovine serum conglutinin, and bovine serum collectin 43 (CL-43). These proteins are structurally similar and share the same domain organization (Hansen and Holmskov, 1998). Although not a lectin, Clq also shares a similar common structure except it has a globular head group that recognizes immunoglobulins rather than oligosaccharides (Lu et al., 1993).

1.5.4.2 Activation of Lectin Pathway

Mannose-binding lectin (MBL) is a classical complement pathway activator (Ohta et al., 1990; Lu et al., 1990; Ikeda et al., 1987). This complement activation pathway is also referred to as the lectin pathway. MBL can activate complement upon binding to carbohydrate residues on micro-organisms. Binding studies have shown that the calcium-
dependent carbohydrate-recognition domain of MBL binds only to sugar containing an equatorial 4-OH group (e.g. N-acetylmannosamine, mannose, N-acetylglucosamine and glucose) but not sugar containing axial 4-OH (e.g. N-acetyl galactosamine and galactose) (Elgavish and Shaanan, 1997; Kawasaki et al., 1989). The affinities of MBL for the different sugar residues in decreasing order are as followed: N-acetylglucosamine > mannose, N-acetylmannosamine, and fucose > maltose > glucose >> galactose and N-acetylgalactosamine (Holmskov et al., 1994). The binding of a single carbohydrate-recognition domain to a saccharide has a relatively low $K_d$ of $10^{-5}$ to $10^{-3}$ M. In contrast, the affinity of the oligomeric MBL to mannan is $K_d$ $10^{-9}$ M because of the increased avidity provided by the molecule's oligomeric structure (Kozutsumi et al., 1980). Upon binding to carbohydrate structures, MBL activates MBL-associated serine protease 1 (MASP-1) and MASP-2. The activation of these two MASPs will enzymatically activate C4 and C2 and lead to the activation of the classical complement pathway (Thiel et al., 1997; Matsushita and Fujita, 1992).

MBL is a C-type lectin that has important roles in the innate immune system, which is critical in the first line of host defense such as bactericidal activity and opsonization. MBL has been demonstrated to bind rough strains of *E. coli* (Kawasaki et al., 1989), *Candida albicans* (Tabona et al., 1995), HIV-1 and HIV-2 (Haurum et al., 1993), influenza A (Anders et al., 1994; Hartshorn et al., 1993), *Salmonella montevideo* (Kuhlman et al., 1989), *Listeria monocytogenes*, *Haemophilus influenzae*, *Neisseria meningitidis*, and *Neisseria cinera* (van Emmerik et al., 1994).

MBL may be involved in rheumatoid arthritis (Malhotra et al., 1995). Autoantibodies of IgG isotype that developed in rheumatoid arthritis undergo
glycosylation changes to unmask terminal N-acetylglucosamine residue in the Fc region for MBL binding. MBL may bind to these IgGs and activate complement.

1.5.4.3 MBL Deficiency

Most MBL deficiencies are linked to structural mutations in codon 54 (G → D) (Sumiya et al., 1991) or codon 57 (G → E) (Madsen et al., 1994; Lipscombe et al., 1992). It is believed that the substitutions of glycine residues with aspartate or glutamate, which have more bulky R-groups, interfere with the formation of the collagen-like domain and lead to lower serum MBL level. A third mutation in codon 52 (R → C) is also linked to MBL deficiency, but with less effect on MBL level (Madsen et al., 1994).

MBL deficiency is associated with an opsonic defect (Garred et al., 1995; Super et al., 1989) and a phagocytic defect (Garred et al., 1995). Deficiencies of MBL also predispose individuals to SLE (Davies et al., 1997; Sullivan et al., 1996; Lau et al., 1996). Recently, associations between MBL deficiency and recurrent fetal wastage have been also observed in different studies; deficiency of MBL in maternal serum is associated with unexplained recurrent miscarriage (Christiansen et al., 1999; Kilpatrick et al., 1995) and preeclampsia (Kilpatrick, 1996).

1.5.5 Complement and Antiphospholipid Antibodies

Elevated levels of complement activation (Hammond et al., 1989) and hypocomplementemia have been reported in SLE patients with APS (Arvieux et al., 1991; Hazeltine et al., 1988). In vitro complement activation on the platelet surface in
the presence of aPL due to C5b-9 deposition has been demonstrated (Stewart et al., 1997).

1.6 Rationale and Overall Objectives

β2GPI is one of the most common targets of aPL. The adsorption of β2GPI to an oxidized surface is necessary to express neo-epitope(s) for aPL binding (Chamley et al., 1999; Ichikawa et al., 1994; Matsuura et al., 1994; Roubey, 1994; Kandiah and Krilis, 1994; Arvieux et al., 1991). Strong associations between clinical thrombosis and the binding of aPL to surface-associated β2GPI also exist (Day et al., 1998; Swadzba et al., 1997; Pengo et al., 1996; Cabiedes et al., 1995). These observations lead to the hypothesis that surface-bound β2GPI may undergo conformation changes and express important sites of interaction with proteins involved in maintaining hemostasis, which are then disrupted upon aPL binding. Therefore, the possible interactions between neo-epitope(s) expressed by β2GPI and other hemostatic proteins were investigated.

The objective of this project was to understand the interactions of phospholipid-bound or oxidized surface-bound β2GPI with the anticoagulation mechanism as well as with the complement system in the setting of antiphospholipid syndrome. There were four specific aims of this thesis. (1) The interaction of β2GPI and APC was studied in the presence of different phospholipids and protein S to examine the effect of their interactions in APC anticoagulation activity. (2) The tri-molecular interactions among β2GPI, protein S, and C4BP were characterized to elucidate the significance of these interactions in protein S anticoagulation activity. (3) The effects of aPL on β2GPI
interactions with APC as well as protein S were studied to determine whether these interactions would be interfered with by aPL and lead to thrombogenesis. (4) The complement activation in the presence of β2GPI-bound aPL and the interactions of β2GPI with complement proteins C4BP and MBL were investigated to examine the role of β2GPI and aPL in complement activation.
CHAPTER 2 MATERIALS AND METHODS

2.1 Reagents

Human β2GPI (Wurm, 1984), protein S (Dahlback, 1983b), C4BP, and C4BP (Dahlback, 1983a) were purified as described. Mannose-Sepharose and maltose-Sepharose were prepared using a previously described method (Fornstedt and Porath, 1975). APC, protein C, protein S, and C4BP were obtained from Haematologic Technologies (Essex Junction, VT). Bovine serum albumin (BSA) was purchased from Sigma (Oakville, ON, Canada). Human C4b, and non-immunogenic rabbit IgG were obtained from Cedarlane (Hornby, ON, Canada). Goat anti-human protein C, sheep anti-human protein S, goat anti-human C4BP, affinity-purified rabbit-anti-human β2GPI, monoclonal mouse-anti-human protein S, and monoclonal mouse-anti-human C4BP were purchased from Enzyme Research Laboratories (South Bend, IN). Horseradish peroxidase (HRP)-conjugated donkey antibodies against human IgG, goat IgG, sheep IgG, mouse IgG, and fluorescein isothiocyanate (FITC)-F(ab’)2 donkey anti-mouse IgG were obtained from Jackson ImmunoResearch (West Grove, PA). FITC-labeled mouse-anti-human CD62P was purchased from Beckman-Coulter (Miami, FL). Monoclonal mouse-anti-human C5b-9 (clone aE11) was purchased from Dako (Mississauga, ON, Canada). Monoclonal mouse-anti-human MBL (clone 131) was provided by Dr. Steffen Thiel (University of Aarhus, Aarhus, Denmark). Phosphatidylcholine (PC), phosphatidylethanolamine (PE), and phosphatidylserine (PS) were purchased from Avanti Polar Lipids (Birmingham, AL). Chromogenic substrate S-2366 (L-
pyroglutamyl-L-prolyl-L-arginine-p-nitroaniline) was purchased from Chromogenix (DiaPharma, West Chester, OH).

2.2 Purification of MBL

Human MBL was purified as according to Tan et al. (1996) with modification. Human serum (Canadian Blood Services, Vancouver, Canada) was used as starting material. A mannose-Sepharose column and a maltose-Sepharose column were used sequentially to isolate crude MBL. The crude MBL obtained after these two column steps was further purified using Sephacryl S-300 equilibrated in an acetate buffer (100 mM sodium acetate, 100 mM NaCl, 5 mM EDTA, pH 5.0) to remove MBL-associated serine protease (MASP).

2.3 Normal Human Plasma Preparation

Pooled normal human plasma (NHP) was prepared from 15 female and 10 male healthy volunteers. Blood was collected into Vacutainer (Becton-Dickinson, Rutherford, NJ) tubes containing 3.8 % sodium citrate, centrifuged at 2000 x g for 15 min. The plasma was pooled and stored in aliquots at -80 °C.

2.4 Normal Human Serum Preparation

Pooled normal human serum (NHS) was prepared from blood collected from healthy donors. Blood was collected into whole blood Vacutainer (Becton-Dickinson, Rutherford, NJ). The tubes were left at room temperature (RT) for 30 min for clotting
and were then centrifuged at 2000 \times g for 15 min. The serum was removed, pooled and stored in aliquots at \(-80^\circ C\).

### 2.5 Blood Samples

Consecutive blood samples were collected from patients who were tested positive or suspected to be positive for anticardiolipin with informed consent from the patients. Ethics approval was obtained from the University of British Columbia Committee for the Use of Human Subjects in Research. Normal samples were collected from healthy volunteers.

### 2.6 Detection of Anti-Cardiolipin and Anti-\(\beta_2\)GPI Using ELISA

The anti-cardiolipin IgG and IgM levels for patient and normal samples were measured using TheraTest EL-ACA enzyme immunoassay (DiaSorin, Stillwater, MN). Samples with IgG > 15 GPU or IgM > 10 MPU were considered as positive. GPU and MPU stand for IgG phospholipid units and IgM phospholipid units, respectively. One GPU is the binding activity of 1 \(\mu g/mL\) aCL IgG (Harris et al., 1987). MPU has the same definition for aCL IgM. The anti-\(\beta_2\)GPI IgG and IgM levels were measured using QUANTA Lite \(\beta_2\)GPI IgG and QUANTA Lite \(\beta_2\)GPI IgM (INOVA Diagnostics, San Diego, CA). Samples with IgG > 20 SGU or IgM > 20 SMU were considered as positive. SGU and SMU stand for standard IgG anti-\(\beta_2\)GPI units and standard IgM anti-\(\beta_2\)GPI units, respectively. One SGU is the binding activity of 1 \(\mu g/mL\) anti-\(\beta_2\)GPI IgG. SMU has the same definition for anti-\(\beta_2\)GPI IgM.
2.7 Isolation and Characterization of IgG Pools

Samples from 10 patients positive for IgG antiphospholipid antibodies (35 to >150 GPU) and a history of thrombosis were pooled and the IgG purified using protein G-Sepharose according to the manufacturer's published protocol (Amersham Pharmacia Biotech, Piscataway, NJ). Normal human IgG (NHIgG) was similarly isolated from a normal human plasma pool of 25 healthy volunteers with normal aPL levels (<15 GPU).

2.8 Specificities of IgG Pools

High binding microtitre wells (Costar Corning, NY) were coated with 100 μL (10 μg/mL) of β2GPI, APC, protein S, or C4BP in Tris-buffered saline (TBS; 50 mM Tris, 150 mM NaCl, pH 7.4) overnight at 4°C and blocked with TBS-CaM (TBS containing 2.5 mM CaCl₂, 5% w/v skim milk powder, pH 7.4). Wells were washed five times with TBS-TCa (TBS containing 0.05% Tween 20 and 2.5 mM CaCl₂, pH 7.4). One hundred microliters (0.5 mg/mL) of pooled aPL or NHIgG in TBS-CaM were added to the wells and incubated with shaking (360 rpm) for 3 hr at room temperature (RT). Wells were washed five times with TBS-TCa and bound IgG was detected using horseradish peroxidase-anti-human IgG and 3,3',5,5'-tetramethylbenzidine (TMB) substrate at OD₄₅₀.

2.9 ¹²⁵I-Radiolabeling of Proteins

APC, protein C, protein S, C4BP, MBL, β2GPI, and BSA were radiolabeled with Na¹²⁵I (Amersham Pharmacia Biotech) using the Iodogen (Pierce, Rockford, IL) method. A glass test tube was pre-coated with 10 μg of iodogen. One hundred to two hundred micrograms of APC, protein C, protein S, C4BP, MBL, β2GPI, or BSA were incubated
with 100 μCi of Na$^{125}$I in the iodogen-coated tube for 10 min at RT. An aliquot of the protein was precipitated with trichloroacetic acid (TCA) and spun down by centrifugation. The relative amounts of $^{125}$I in the protein precipitate pellet and in the supernatant were counted to determine the percent incorporation of $^{125}$I in protein using LKB Wallac gamma counter (Compugamma model 1282). Separation of free $^{125}$I from $^{125}$I-protein was achieved by passing the reaction mixture through a PD-10 size exclusion column (Amersham Pharmacia). Fractions with more than 98% of the $^{125}$I incorporated into the protein as determined by TCA precipitation were pooled. The concentration of $^{125}$I-labeled protein was determined using BCA protein assay (Pierce, Rockford, IL) with the unlabeled protein counterpart as standard.

2.10 Effects of Co-Incubating β2GPI and Protein S on APC Activities in Phosphatidylserine-Coated Wells

High-binding microtitre plates (Costar Corning, NY) containing 100 μL/well of PS (50 μg/mL in 100% ethanol) were air-dried at room temperature and blocked with TBS-CaM. After washing with TBS-Ca (TBS, 2.5 mM CaCl$_2$, pH 7.4), 100 μL of $^{125}$I-trace labeled APC (2 μg/mL, 32 nM) in TBS-Ca in the presence of 1) β2GPI (100 μg/mL, 2 μM), 2) protein S (10 μg/mL, 145 nM), or 3) β2GPI (100 μg/mL, 2 μM) and protein S (10 μg/mL, 145 nM) were incubated at 37°C for 2 hours in PS-coated wells. After the incubation, wells were washed with ice-cold TBS-Ca four times. One hundred microliters of chromogenic substrate S-2366 (1 mM in TBS-Ca buffer) were added and incubated for 60 min at RT. The amount of substrate hydrolyzed over time was measured by OD$_{405}$. The amounts of APC bound were measured with a Gamma Counter. The
APC activities in PS-coated wells with different amount of APC bound were also measured by incubating PS-wells with 0 to 2 μg/mL of APC.

2.11 Effects of β2GPI on APC Activities in Phosphatidylcholine-, Phosphatidylethanolamine-, Phosphatidylserine-Coated Wells

Microtitre wells containing 100 μL/well of PC, PE, or PS (50 μg/mL in 100% ethanol) were air-dried at room temperature and blocked with TBS-CaM. After washing with TBS-Ca, 100 μL of $^{125}$I-trace labeled APC (2 μg/mL, 32 μM) with or without β2GPI (100 μg/mL, 2 μM) in TBS-Ca were incubated at 37°C for 2 hours. The amount of APC activity and phospholipid-bound APC were measured by hydrolysis of chromogenic substrate S-2366 and gamma-counting, respectively, as above.

2.12 Interactions of APC, Protein C, Protein S, C4BP, C4BP₂, and MBL with ELISA Plate-Bound β2GPI Under Different Conditions

High-binding microtitre wells were coated with 100 μL β2GPI (10 μg/mL) in TBS overnight at 4°C or 2 hr at RT and blocked with TBS-CaM; wells coated only with TBS-CaM served as controls. After washing with TBS-TCa, 100 μL of APC, protein C, protein S, C4BP, C4BP₂, or MBL in TBS-CaM (0 to 10 μg/mL) was added and incubated with shaking (360 rpm) for 3 hr at RT. After washing five times with TBS-TCa, 100 μL of goat anti-human protein C, sheep anti-human protein S, goat anti-human C4BP, or mouse-anti-human MBL in TBS-CaM were added for an hour incubation. Wells were washed five times with TBS-TCa and bound IgG was visualized using appropriate HRP-conjugated secondary antibodies in TBS-CaM and TMB substrate.
The effects of fluid-phase β2GPI on the interactions between ELISA plate-bound β2GPI and fluid phase APC, protein S, and C4BP were studied similarly by co-incubating APC, protein S, or C4BP (0 to 10 μg/mL) with β2GPI (50 μg/mL) in β2GPI-coated wells. The effect of fluid-phase C4b (50 μg/mL) on C4BP (0 to 10 μg/mL) binding to ELISA plate-bound β2GPI was also investigated by pre-incubating the C4b-C4BP mixture for 2 hours before adding to ELISA plate coated with β2GPI. The amount of protein binding was measured using the protocol above.

The competitive bindings of APC, protein S, and C4BP to β2GPI when they were in the presence of each other were studied similarly by co-incubating 1) APC (0 to 10 μg/mL) and protein S (10 μg/mL), 2) protein S (0 to 10 μg/mL) and APC (10 μg/mL), 3) protein S (0 to 10 μg/mL) and C4BP (200 μg/mL), or 4) C4BP (0 to 10 μg/mL) and protein S (20 μg/mL) in β2GPI-coated wells for 3 hours at RT.

The same procedure was also used to study the effects of aPL IgG (500 μg/mL) on APC, protein S, and C4BP (0 to 10 μg/mL) binding to β2GPI. In addition, different amounts of aPL IgG (0 to 1 mg/mL) were incubated with APC, protein S, or C4BP (5 μg/mL) in β2GPI-coated wells to study the dose effects of aPL IgG on these β2GPI-dependent interactions. NHIgG (500 μg/mL) was used as control.

The effects of aPL IgG (1 mg/mL) and N-acetylmannosamine (0.2 M) on MBL binding to β2GPI were examined similarly with NHIgG (1mg/mL) and galactose (0.2M) as controls respectively.

The binding of APC, protein S, C4BP, and MBL to β2GPI in the presence of 10 mM EDTA was also examined. One hundred microliters of APC, protein S, C4BP, or MBL (0 to 10 μg/mL) in TBS-EDTAM (TBS, containing 10 mM EDTA and 5% w/v
powdered skim milk, pH 7.4) was incubated with shaking (360 rpm) for 3 hr at RT in wells coated with β2GPI as above. After the incubation, microtitre wells were washed three times with TBS-TEDETA (TBS containing 0.05% Tween 20 and 10 mM EDTA, pH 7.4) and two times with TBS-TCa. The amount of APC, protein S, C4BP, or MBL bound was measured using the condition as above.

For all assays, the amount of protein binding was reported after subtracting OD$_{450}$ of wells without added APC, protein C, protein S, C4BP, C4BP$_{a}$, or MBL.

2.13 Effects of High Binding ELISA Plate-Bound β2GPI on APC Activities

Microtiter wells were coated with 100 μL of β2GPI (10 μg/mL) in TBS overnight at 4°C and blocked with TBS-CaM. After washing with TBS-Ca, 100 μL of $^{125}$I-trace labeled APC (10 μg/mL) in TBS-Ca were incubated at 37°C for 2 hours. The APC activities in PS-coated wells with different amount of APC bound were used as standard. The amount of APC activity and phospholipid-bound APC were measured by hydrolysis of chromogenic substrate S-2366 and gamma-counting respectively as above.

2.14 Kinetics and Equilibrium Dissociation Constants of APC, Protein C, Protein S, C4BP, MBL, and BSA Interactions with β2GPI

Microtiter wells were coated with 100 μL of $^{125}$I-trace labeled β2GPI (10 μg/mL) in TBS overnight at 4°C and blocked with TBS-CaM. After washing with TBS-Ca, 100 μL of $^{125}$I-trace labeled APC, protein C, protein S, C4BP, MBL, or BSA (10 μg/mL) in TBS-Ca were incubated at 37°C. Proteins were removed and wells were washed with ice-cold TBS-Ca four times at different time points, up to 300 minutes. The amounts of
APC, protein C, protein S, C4BP, MBL, and BSA bound to β2GPI were quantified by gamma-radiation counting.

The equilibrium dissociation constants for APC-, protein C-, protein S-, C4BP-, MBL-, and BSA-β2GPI interactions were determined by incubating $^{125}$I-trace labeled APC, protein C, protein S, MBL, BSA (0 - 80 µg/mL), or C4BP (0-800 µg/mL) in wells previously coated with 100 µL $^{125}$I-trace labeled β2GPI (10 µg/mL) for 4 hours at 37°C. Proteins were removed and wells were washed with ice-cold TBS-Ca four times. The amounts of protein bound were measured by gamma-radiation counting. Binding constants were determined by non-linear curve fitting using Origin software version 6.0 (Microcal, Northampton, MA). Binding assays using only labeled proteins or mixtures of labeled proteins and unlabeled proteins were also carried out similarly to examine the effects of radiolabeling on equilibrium dissociation constants.

2.15 Competitive ELISA For Determining Anti-Protein C Antibody Specificity For APC and Protein C

Microtitre plates were coated with 100 µL APC (10 µg/mL) in TBS overnight at 4°C or 2 hr at RT and blocked with TBS-CaM. After washing with TBS-TCa, 100 µL of APC or protein C (0 to 50 µg/mL) in TBS-CaM containing goat-anti-protein C antibodies (1/1000 dilution) was added and incubated with shaking (360 rpm) for 3 hr at RT. The amount of goat-anti-protein C bound was measured using the ELISA protocol above. This experiment was also performed using protein C-coated microtitre plates.
2.16 Effects of Anti-β2GPI Sepharose Beads and β2GPI-Sepharose Beads on Plasma Protein S Clotting Activity

Anti-β2GPI Sepharose beads were prepared by coupling 2 mg of affinity-purified rabbit-anti-human β2GPI to 1 mL of CNBr-Sepharose 4B packed gel beads. For the experiment, two hundred microliters of NHP was added to 1 mL of packed anti-β2GPI Sepharose beads equilibrated in Owren-Koller buffer (0.03 M sodium barbitone, 0.1 M sodium acetate, pH 7.4). This gel slurry was incubated for 30 minutes at 4°C on a rotator. NHP was eluted and the concentration was adjusted to one-tenth of the initial NHP concentration with Owren-Koller buffer. The activity of free protein S remained in NHP after being exposed to anti-β2GPI gel beads was assessed using Staclot Protein S kit (Diagnostica, Stago, France) according to the manufacturer’s instructions on a Stago STA coagulation analyzer. Briefly, 50 μL of the 10 times diluted anti-β2GPI-gel beads treated NHP was incubated at 37°C for 2 min with 50 μL of each of the following reagents: protein S-deficient human plasma, human activated protein C, and a preparation containing bovine factor Va. The time to form a clot was measured after the addition of 50 μL of 0.025 M CaCl₂. The time for clotting is translated into a percentage of the NHP control based on a standard curve which is linear between 25-120 %. NHP treated with non-immune rabbit IgG-gel beads and NHP not exposed to gel beads were used as controls. The effect of incubating NHP with β2GPI-conjuated Sepharose beads on protein S activity was also studied similarly. β2GPI-conjuated Sepharose beads were prepared by coupling 4 mg of β2GPI to 1 mL of CNBr-Sepharose 4B packed gel beads.
2.17 Effects of aPL IgG on APC Activities in the Presence of β2GPI and Protein S in PS-Coated Wells

High-binding microtitre plates containing 100 μL/well of PS (50 μg/mL in 100% ethanol) were air-dried at room temperature and blocked with TBS-CaM. After washing with TBS-Ca, 100 μL of protein mix containing 1) APC + β2GPI + NHIgG, 2) APC + β2GPI + aPL IgG, 3) APC + β2GPI + NHIgG + protein S, and 4) APC + β2GPI + aPL IgG + protein S were added. The concentrations of the 125I-trace labeled APC, β2GPI, aPL IgG, and NHIgG used were 2 μg/mL, 100 μg/mL, 1 mg/mL, and 1 mg/mL, respectively. After the incubation, wells were washed with ice-cold TBS-Ca four times. One hundred microliters of chromogenic substrate S-2366 (1 mM in TBS-Ca buffer) was added and incubated for 60 min at RT. The amount of substrate hydrolyzed over time was measured by OD405. The amounts of APC bound were measured by gamma-radiation counting. The APC activities in PS-coated wells with different amount of APC bound by incubating PS-wells with 0 to 2 μg/mL of APC were also measured.

2.18 Effects of aPL IgG on β2GPI Binding to PS-Coated ELISA Wells

PS-coated ELISA plates were prepared as above. After washing with TBS-Ca, 100 μL of protein mix containing 1) 125I-β2GPI (0 to 10 μg/mL) and NHIgG (500 μg/mL) and 2) 125I-β2GPI (0 to 10 μg/mL) and aPL IgG (500 μg/mL) were added. After the incubation, wells were washed with ice-cold TBS-Ca four times. The amounts of APC bound were measured by gamma-radiation counting.
2.19 Effects of aPL IgG on APC, Protein S and C4BP Binding to Activated Platelets in the Presence of β2GPI

Washed platelets were prepared from citrated blood samples collected from healthy volunteers. Platelet-rich plasma, diluted 1:1 with acid-citrate-dextrose, was centrifuged at 2000 x g for 15 min, resuspended in MHTB (modified HEPES Tyrode’s Buffer; 10 mM HEPES, 137 mM NaCl, 2.7 mM KCl, 0.4 mM NaH$_2$PO$_4$, 0.1% dextrose, 0.2% BSA, pH 6.5), and centrifuged at 2000 x g for another 10 min. The final platelet pellets were resuspended in MHTB-Ca (MHTB, 2.5 mM CaCl$_2$, pH 7.4). Washed platelets ($2 \times 10^7$/mL) were incubated with β2GPI (200 μg/mL), APC (4 μg/mL), protein S (20 μg/mL), gly-pro-arg-pro (25 μM; Novabiochem, San Diego, CA), and aPL IgG (1 mg/mL) in MHTB-Ca buffer and activated by calcium ionophore (A23187) (10 μg/mL; Sigma). NHIgG (1 mg/mL) in place of aPL IgG and resting platelets in place of activated platelets were used as controls. After 1 hr of incubation at 37°C, platelets were washed with MHTB-Ca and incubated with FITC-labeled monoclonal anti-human CD62P to monitor activation state, or unlabeled monoclonal anti-human protein C or anti-human protein S. After 30 min, platelets were washed and incubated with FITC-labeled F(ab')$_2$ donkey anti-mouse IgG for the anti-human protein C and the anti-human protein S incubations for another 30 min then diluted in TBS prior to performing flow cytometry. The effects of aPL on protein S and C4BP binding to platelets were studied using an identical procedure except APC and monoclonal anti-protein C were replaced by C4BP (250 μg/mL) and monoclonal anti-human C4BP, respectively.
2.20 Binding of $^{125}$I-$\beta$2GPI and $^{125}$I-Protein S to Activated Platelets in the Presence of aPL

Washed platelets (4 x $10^7$/mL) were incubated with $^{125}$I-trace labeled $\beta$2GPI (200 µg/mL), gly-pro-arg-pro (25 µM; Novabiochem, San Diego, CA), and aPL IgG (1 mg/mL) in MHTB-Ca buffer and activated by calcium ionophore (A23187) (10 µg/mL) in a final volume of 50 µL. NHIgG (1 mg/mL) in place of aPL IgG and resting platelets in place of activated platelets were used as controls. After 1 hr of incubation at 37°C, platelet samples were made up to 1000 µL in MHTB-Ca and centrifuged at 2000 x g for 15 min. Nine hundred microliters of supernatant were transferred to another tube (tube #2), leaving 100 µL of supernatant together with the platelet pellet (tube #1). The radioactivity bound to platelets in each sample was calculated using the following formula:

$$\text{Binding} = \frac{(\text{tube } #1) - (\text{tube } #2)}{9}$$

The binding of $^{125}$I-trace labeled $\beta$2GPI (200 µg/mL) and $^{125}$I-trace labeled protein S (20 µg/mL) to activated washed platelets without IgGs was measured similarly.

2.21 ELISA To Detect Antiphospholipid Antibody-Mediated Complement Activation

ELISA plates were coated with 100 µL $\beta$2GPI (10 µg/mL) in TBS overnight at 4°C or 2 hr at RT and blocked with GVB$^{2+}$ (VBS, [veronal buffered saline, containing 1.8 mM sodium barbitone, 1.56 mM sodium barbituric acid, 145 mM NaCl], and 0.15 mM CaCl$_2$, 1 mM MgCl$_2$, 0.1 % gelatin, pH 7.4); wells coated only with GVB$^{2+}$ served as
controls. After washing with GVB\textsuperscript{2+}, 100 µL of positive controls containing serially diluted rabbit-anti-human β2GPI and 1:1 diluted patient serum samples in GVB-EDTA (VBS containing 20 mM EDTA, 0.1 % gelatin, pH 7.4) were added and incubated for 1 hr at RT. After washing three times with GVB-EDTA, and three times with GVB\textsuperscript{2+}, 100 µL of NHS diluted 1:40 in GVB\textsuperscript{2+} were added to the wells and incubated at 37°C for 1 hr for complement activation. After the incubation, wells were washed 5 times with TBS-TCa and incubated with monoclonal mouse anti-human C5b-9 in GVB\textsuperscript{2+} for 1 hr. Wells were washed five times with TBS-TCa and mouse anti-human C5b-9 was visualized using HRP-donkey-anti-mouse IgG in GVB\textsuperscript{2+} and TMB substrate. Non-specific mouse IgG was used as primary antibody control to ensure signals produce by the ELISA were due to specific anti-C5b-9 antibody binding to ELISA plate-bound C5b-9. Controls without primary antibodies were also performed. Controls in which β2GPI- and GVB\textsuperscript{2+}-coated wells were incubated with serum in GVB\textsuperscript{2+} or GVB-EDTA and probed with anti-C5b-9 were performed to examine the ability of β2GPI to mediate complement activation.

2.22 Statistical Analysis

The statistical analyses of the data were performed using t-test, paired t-test, ANOVA, 2 way ANOVA, and Newman-Keul’s Test. The calculations were carried out using Microsoft Excel 97 (Redmond, WA).
CHAPTER 3 EFFECT OF $\beta_2$-GLYCOPROTEIN I ON THE SPECIFIC ACTIVITY OF ACTIVATED PROTEIN C IN THE PRESENCE OF AMINOPHOSPHOLIPIDS

$\beta_2$GPI may be a procoagulant APC inhibitor (Mori et al., 1996; Canfield and Kisiel, 1982) as well as a natural anticoagulant (Brighton et al., 1996). Also, several studies have shown that the anticoagulation activity of APC could be inhibited by aPL in the presence of $\beta_2$GPI. However, the mechanism that mediates this inhibition and the role of $\beta_2$GPI in this phenomenon remain unclear. Therefore, the interaction between APC and $\beta_2$GPI was investigated to gain further understanding of their interrelationships.

3.1 Effects of $\beta_2$GPI and Protein S on APC Activity on Phospholipid Surface

The activity of PS-bound APC was quantified by measuring the hydrolysis of chromogenic substrate S-2366. A representative curve of the hydrolysis of S-2366 by different amounts of PS-bound APC as measured by OD$_{405}$ after 60 minutes of incubation is shown in Figure 5. The hydrolysis of S-2366 varied linearly with the amount of APC. This indicates that the measurement of APC activity in the presence of 1 mM S-2366 at room temperature is directly proportional to the amount of APC bound to PS. Therefore, the specific activity of APC can be determined under these experimental conditions.

Using the same experimental conditions, the effect of $\beta_2$GPI on the specific activity of PS-bound APC was assessed. In Figure 6A, the specific activity of PS-bound APC was 18 OD$_{405}$/μg. In comparison, when APC and $\beta_2$GPI were co-incubated in PS-coated wells, the specific activity of PS-bound APC increased 3 fold, to 59 OD$_{405}$/μg (p < 0.001, n=5, ANOVA). The co-incubation of APC and protein S led to the expected
Figure 5. Hydrolysis of Chromogenic Substrate S-2366 by PS-Bound APC.

PS-coated wells were incubated with $^{125}$I-trace labeled APC (0 to 2 µg/mL). The activity of PS-bound APC was quantified by measuring the hydrolysis of chromogenic substrate S-2366 (1 mM) after 60 minutes of incubation at RT. The amounts of APC bound to PS after the incubation were measured by gamma-radiation counting.
Figure 6. Specific Activity of PS-Bound APC in the Presence of β2GPI and Protein S. PS-coated wells were incubated with 1) $^{125}$I-trace labeled APC (2 µg/mL, 32 nM), 2) APC (2 µg/mL, 32 nM) + β2GPI (100 µg/mL, 1.9 µM), 3) APC (2 µg/mL, 32 nM) + protein S (10 µg/mL, 145 nM), or 4) APC (2 µg/mL, 32 nM) + β2GPI (100 µg/mL, 1.9 µM) + protein S (10 µg/mL, 145 nM). (A) The specific activity of APC bound to PS-wells was obtained from the total activity generated by the amount of PS-bound APC. (B) The total activity was quantified by measuring the hydrolysis of chromogenic substrate S-2366 (1 mM) after 60 minutes of incubation at RT with PS-bound APC. Data points are the mean ± 1 SD of five separate measurements. Data are representative of 3 separate experiments.
increase in APC specific activity of 24 OD405/μg, which was only a 1.3 fold increase compared to incubation with APC alone (p < 0.001, n=5, ANOVA). In comparison to APC co-incubation with protein S, when APC was co-incubated with β2GPI and protein S, the PS-bound APC had a specific activity of 59 OD405/μg (p < 0.001, n=5, ANOVA). Controls containing the same protein mixtures except APC showed no APC activity (data not shown). These results suggest that β2GPI can serve as an APC cofactor to increase APC specific activity. Also, purified β2GPI was free of protein S as indicated by the absence of protein S band in silver stained gel analysis (data not shown). Therefore, the increase in APC specific activity in the presence of β2GPI was not due to protein S contamination in the incubation mixture.

This increase in APC specific activity in the presence of β2GPI, however, did not translate into an increase in total APC activity. In Figure 6B, the total activity generated by PS-bound APC was an OD405 of 0.593. When APC and β2GPI were co-incubated together in PS-coated wells, the total activity of PS-bound APC was only 0.462, a 22 % reduction (p < 0.001, n=5, ANOVA). The co-incubation of APC and protein S also led to a slightly lowered total APC activity of 0.568 compared to incubation with APC alone; however this was not statistically significantly. In comparison to APC co-incubation with protein S, when APC was co-incubated with β2GPI and protein S, the PS-bound APC had a total activity of 0.441 (p < 0.001, n=5, ANOVA). Therefore, on a PS surface, β2GPI and protein S lowered APC total activity. This may be because both β2GPI and protein S, similar to APC, are anionic phospholipid binding proteins, and would compete with APC for PS binding. Hence, less APC was bound and less APC activity was detected in their presence.
3.2 Activity of APC Bound to PC-, PE-, and PS-Coated Wells in the Presence of β2GPI

This experiment examined the effect of different phospholipids on APC specific activity. In Figure 7A, the specific activity of PS-bound APC was 18 OD₄₀₅/µg. When APC and β2GPI were co-incubated in PS-coated wells, the specific activity increased to 59 OD₄₀₅/µg (p < 0.001, n=5, t-test). Interestingly, the specific activity of APC bound to the neutral PC was 50.7 OD₄₀₅/µg and β2GPI did not affect the specific activity significantly. This lack of enhancement of APC activity in the presence of β2GPI is probably due to the negligible binding of β2GPI to PC or that the APC activity was already near maximal. The specific activity of PE-bound APC was 31.9 OD₄₀₅/µg and the presence of β2GPI increased the specific activity to 44.9 OD₄₀₅/µg (p < 0.002, n=5, t-test).

Even though PC-bound APC exhibits higher specific activity than PE-bound and PS-bound APC, PC-bound APC probably is a minor or even negligible contributor to APC anticoagulation activity in vivo. In Figure 7B, the total activity of PC-bound APC generated over 60 minutes was an OD₄₀₅ of 0.065, compare to 0.316 and 0.593 for PE-bound and PS-bound APC, respectively. Therefore, PC-bound APC only had 10 to 20 % of the total activity of APC bound to aminophospholipid PE and PS. This is because APC binds strongly to PE and PS, but not PC. Consequently, higher total APC activity was observed in PE- and PS- coated wells even though PE-bound and PS-bound APC have a lower specific activity. When APC was co-incubated with β2GPI in PC-coated
Figure 7. Specific Activity of phospholipid-bound APC in the presence of β2GPI in PC-, PE-, and PS- Coated Wells.

PC-, PE-, and PS-coated wells were incubated with $^{125}$I-trace labeled APC (2 μg/mL, 32 nM) in the presence of absence of β2GPI (100 μg/mL, 1.9 μM). (A) The specific activity of APC bound to phospholipid coated-wells was obtained from the total activity generated by the phospholipid-bound APC and the amount of phospholipid bound APC. (B) The total activity was quantified by measuring the hydrolysis of chromogenic substrate S-2366 (1 mM) after 60 minutes of incubation at RT with PS-bound APC. Data points are the mean ± 1 SD of four separate measurements. Data are representative of 3 separate experiments.
wells, the total activity was 0.0528, slightly lower than incubation with APC alone. In
the presence of B2GPI, the total activities of PE-, and PS-bound APC were reduced to
0.251 (p < 0.003, n=5, t-test) and 0.462 (p < 0.001, n=5, t-test), respectively. These
reductions probably reflect the competition between B2GPI and APC for PE and PS.

3.3 Effect of Fluid Phase B2GPI on APC and Protein S Binding to Oxidized
ELISA Plate-Bound B2GPI in the Absence of Phospholipids

To assess whether the APC-B2GPI interaction was dependent on PE and PS, APC
binding to B2GPI was evaluated in the absence of phospholipid. Using ELISA, in the
presence of 2.5 mM CaCl₂, APC was found to interact with B2GPI adsorbed to oxidized
high-binding plates in a concentration-dependent manner in the absence of phospholipids
(Figure 8A). In the presence of 10 mM EDTA, APC binding to B2GPI was inhibited (p
< 0.001, two way ANOVA). APC did not bind to control wells coated with skim milk
protein. The presence of 50 µg/mL (930 nM) fluid phase B2GPI did not inhibit APC (0
to 10 µg/mL, 0 to 161 nM) binding to B2GPI adsorbed to oxidized high-binding plate (p
> 0.05, two way ANOVA) (Figure 8B). This suggests that fluid phase B2GPI does not
interact with APC and fluid phase B2GPI exhibits a conformation different from ELISA
plate-bound B2GPI. This experiment indicates that the conformational alteration that
B2GPI undergoes when bound to an oxidized surface is important for APC binding.
Using the same experimental setup, protein S was also found to bind B2GPI-coated wells
in a concentration- and calcium-dependent manner but not to control wells coated with
Figure 8. Interaction Between β2GPI and APC.

(A) Interaction Between β2GPI and APC is Calcium-Dependent. Microtitre wells coated with β2GPI were incubated with APC with 2.5 mM CaCl₂ (closed circles) or 10 mM EDTA (open circles). Wells blocked with skim milk protein (crosses) controlled for non-specific binding. The OD₄₅₀ reported represents the amount of APC binding after subtracting the OD₄₅₀ of control wells that did not contain APC. (B) Fluid Phase β2GPI Did Not Affect APC Binding to Oxidized ELISA Plate-Bound β2GPI. Microtitre wells coated with β2GPI were incubated with APC (0 to 10 μg/mL, 0 to 161 nM) in the presence (closed circles) or absence (open circles) of fluid phase β2GPI (50 μg/mL, 930 nM). Data points are the means ± 1 SD of three separate measurements of the same sample. The data are representative of 3 separate experiments. The OD₄₅₀ reported represents the amount of APC binding after subtracting the OD₄₅₀ of control wells that did not contain APC. The OD₄₅₀ measurements under the different conditions are very close so that the symbols overlapped and the error bars are smaller than the symbols.
skim milk protein (p < 0.001, two way ANOVA). The presence of 10 mM EDTA partially inhibited this interaction (Figure 9A). Similar to APC binding to β2GPI adsorbed to an oxidized surface, fluid phase β2GPI (50 μg/mL, 930 nM) did not inhibit protein S (0 to 10 μg/mL, 0 to 145 nM) binding to β2GPI adsorbed to oxidized surface (P >0.05, two way ANOVA) (Figure 9B).

3.4 Activity of β2GPI-Bound APC

In this experiment, the effect of oxidized ELISA plate-bound β2GPI on APC was examined to determine whether oxidized ELISA plate-bound β2GPI has the same potentiating effect on APC specific activity as phospholipid-bound β2GPI. APC was incubated in β2GPI-coated plates and the specific activity of APC bound to β2GPI-coated wells was measured. In Figure 10, it can be seen that the activity of β2GPI-bound APC was significantly lower than the equivalent amount of APC bound to PS-wells (p < 0.001). Therefore, APC has lower activity when bound directly to β2GPI compared to an equivalent amount of APC bound directly to PS. This suggests that β2GPI bound to an oxidized surface has an inhibitory effect on APC while β2GPI bound to phospholipids has a potentiating effect on APC.
Figure 9. Interaction Between β2GPI and Protein S.

(A) Interaction Between β2GPI and protein S is Calcium-Dependent. Microtitre wells coated with β2GPI were incubated with protein S with 2.5 mM CaCl₂ (closed circles) or 10 mM EDTA (open circles). Wells blocked with skim milk protein (crosses) controlled for non-specific binding. The OD₄₅₀ reported represents the amount of protein S binding after subtracting the OD₄₅₀ of control wells that did not contain protein S. (B) Fluid Phase β2GPI Did Not Affect Protein S Binding to Oxidized ELISA Plate-Bound β2GPI. Microtitre wells coated with β2GPI were incubated with protein S (0 to 10 µg/mL, 0 to 145 nM) in the presence (closed circles) or absence (open circles) of fluid phase β2GPI (50 µg/mL, 930 nM). Data points are the means ± 1 SD of three separate measurements of the same sample. The data are representative of 3 separate experiments. The OD₄₅₀ reported represents the amount of protein S binding after subtracting the OD₄₅₀ of control wells that did not contain protein S. The OD₄₅₀ measurements under the different conditions are very close so that the symbols overlapped and the error bars are smaller than the symbols.
Protein S (µg/mL)

A

OD_{450}

* p < 0.001

B

OD_{50}

0 5 10 15

Protein S (µg/mL)
Figure 10. Activity of β2GPI-Bound APC.

Microtiter wells coated with β2GPI (closed circles) were incubated with $^{125}$I-trace labeled APC (10 μg/mL). PS-coated wells (open circles) incubated with $^{125}$I-trace labeled APC (0 to 10 μg/mL) were used as controls. The total activity was quantified by measuring the hydrolysis of chromogenic substrate S-2366 (1 mM) after 60 minutes of incubation at RT with β2GPI or PS-bound APC. The amounts of APC bound were measured by gamma-radiation counting. Data points are the mean ± 1 SD of three separate measurements. Data are representative of 3 separate experiments.
3.5 Inhibition of APC Binding to β2GPI by Protein S

Since both APC and protein S could interact with oxidized surface-bound β2GPI, their tri-molecular interactions were examined. The binding of APC (0 to 10 μg/mL, 0 to 160 nM) to β2GPI was inhibited by the presence of 10 μg/mL (145 nM) of protein S (p < 0.001, two way ANOVA). However, the presence of 10 μg/mL of APC did not affect protein S (0 to 10 μg/mL, 0 to 145 nM) binding to β2GPI (Figure 11). These observations suggest that protein S can regulate APC binding to β2GPI whereas APC does not interfere with protein S binding to β2GPI. This observation, therefore, also suggests protein S may serve to prevent oxidized surface bound-β2GPI from inhibiting APC activity by perturbing APC binding to β2GPI.

3.6 Equilibrium Dissociation Constant Determinations for APC, Protein C, Protein S and BSA Binding to β2GPI

To determine the equilibrium dissociation constants for APC, protein C, protein S and BSA binding to β2GPI adsorbed to high-binding ELISA plates, the time required for the protein binding to reach equilibrium was measured. Binding of 10 μg/mL of 125I-trace labeled- APC, protein C, protein S and BSA to β2GPI reached equilibrium after 60 min (Appendix Figure 3, Appendix Figure 4, Appendix Figure 5). The amount of binding of APC, protein C, protein S, or BSA to β2GPI was determined by incubating 125I-trace labeled APC, protein C, protein S, and BSA (0 to 80 μg/mL) for 4 hours at
Figure 11. Inhibition of APC Binding to \( \beta 2\)GPI by Protein S.

Microtitre wells coated with \( \beta 2\)GPI were incubated with: (A) 0 to 10 \( \mu \)g/mL (0 to 160 nM) APC with (open circles) or without 10 \( \mu \)g/mL (145 nM) protein S (closed circles); (B) 0 to 10 \( \mu \)g/mL (0 to 145 nM) protein S with (open circles) or without 10 \( \mu \)g/mL (160 nM) APC (closed circles). Data points are the mean \( \pm \) 1 SD of three separate measurements of the same sample. Data are representative of 3 separate experiments. The OD\(_{450}\) reported represents the amounts of APC and protein S binding after subtracting the OD\(_{450}\) of control wells that did not contain APC and protein S, respectively.
37°C. The equilibrium dissociation constants and molar ratios of the binding were assessed (Table 2). The APC-β2GPI interaction had a $K_d$ of $8.72 \pm 1.64 \times 10^{-7}$ M and an APC:β2GPI molar ratio of $1.5 \pm 0.2$. The protein C-β2GPI interaction had a similar $K_d$ of $7.77 \pm 2.76 \times 10^{-7}$ M and a protein C:β2GPI molar ratio of $1.7 \pm 0.4$. Binding assays indicated that APC and protein C bind β2GPI with very similar dissociation constants with coefficient of variance of 19% and 36%, respectively. The protein S-β2GPI interaction had a $K_d$ of $7.35 \pm 1.66 \times 10^{-7}$ M and a protein S:β2GPI of molar ratio $2.9 \pm 0.4$. The binding of BSA to β2GPI was measured as control. There was negligible interaction, and the association of the two proteins was too weak to measure the equilibrium dissociation constant determination using this experimental setup. Binding assays with only labeled proteins or mixtures of labeled and unlabeled proteins of different ratios also provided comparable binding constants (Appendix Figure 8).

### 3.7 Comparison of Protein C and APC Binding to β2GPI APC Using ELISA

To examine whether APC and protein C have different affinities towards β2GPI, an anti-protein C ELISA was used because of the relatively low coefficient of variance of the ELISA setup. APC was found to bind β2GPI significantly more strongly than its zymogen counterpart, protein C ($P < 0.001$, two way ANOVA) (Figure 12). Anti-protein C antibodies were used to detect the amount of APC and protein C bound to β2GPI-coated plate in this experiment. To ensure that the difference observed was not due to the difference in antibody specificity for APC and protein C, the specificity of anti-protein C
Table 2. Equilibrium Dissociation Constant Determinations for APC, Protein C, and Protein S Binding to β2GPI.

<table>
<thead>
<tr>
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<th>$K_d$ (M)</th>
<th>N/n (mol of protein/mol of β2GPI)</th>
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<tbody>
<tr>
<td>APC</td>
<td>$8.72 \pm 1.64 \times 10^{-7}$</td>
<td>$1.5 \pm 0.2$</td>
</tr>
<tr>
<td>Protein C</td>
<td>$7.77 \pm 2.76 \times 10^{-7}$</td>
<td>$1.7 \pm 0.4$</td>
</tr>
<tr>
<td>Protein S</td>
<td>$7.35 \pm 1.66 \times 10^{-7}$</td>
<td>$2.9 \pm 0.4$</td>
</tr>
</tbody>
</table>
Figure 12. APC and Protein C Binding to β2GPI.

Microtitre wells coated with β2GPI were incubated with APC (closed circles) or protein C (open circles) with 2.5 mM CaCl₂. Data points are the mean ± 1 SD of three separate measurements of the same sample. The data are representative of 3 separate experiments. The OD₄₅₀ reported represents the amount of APC or protein C binding after subtracting the OD₄₅₀ of control wells that did not contain APC or protein C, respectively. Most error bars are smaller than the symbols.

![Graph showing APC and Protein C binding](image-url)
for APC or protein C was determined. Using a competitive ELISA, fluid phase APC and protein C was shown to inhibit anti-protein C binding to ELISA plate-bound APC by the same amount. This indicates the anti-protein C has the same specificity for APC and protein C. Similar results were obtained using a protein C-coated ELISA plate (Figure 13). Therefore, the difference in APC and protein C binding to b2GPI was due to the difference in dissociation constants and not due to the difference in the specificity of anti-protein C for the different proteins.

3.8 Summary

In summary, b2GPI interacted with APC and protein S. b2GPI potentiated APC specific activity on the aminophospholipids PE and PS, but not PC. This enhancement of APC specific activity indicated that there could be a direct interaction between b2GPI and APC. This direct interaction was independent of aminophospholipid as APC could bind directly to b2GPI adsorbed to oxidized ELISA plate. Also, APC binding to oxidized surface-bound b2GPI was not inhibited by fluid phase b2GPI. This suggested that oxidized surface-bound b2GPI expressed a conformation different from the fluid phase b2GPI to support APC binding. Protein S could inhibit APC binding to b2GPI adsorbed to an oxidized surface. Therefore, protein S may be involved in the regulation of the potentiation of APC specific activity by b2GPI on aminophospholipids.
Figure 13. Competitive ELISA For Determining Anti-Protein C Antibodies Specificities For APC and Protein C.

Zero to 50 μg/mL of APC (closed circles) or protein C (open circles) with goat-anti-protein C antibodies (1/1000 dilution) were incubated in microtitre plates were coated with: (A) APC and (B) protein C. The amount of anti-protein C binding to APC- or protein C-coated plate was measured. The data are representative of 3 separate experiments.
CHAPTER 4 B2GPI INTERACTIONS WITH C4BP AND PROTEIN S

B2GPI can down-regulate protein S-C4BP complex formation (Merrill et al., 1999; Atsumi et al., 1997). The mechanism of this tri-molecular interaction was further characterized and confirmed here by investigating the interactions of the protein S-C4BP system with B2GPI bound to oxidized surface or Sepharose beads in both a purified protein system and in plasma.

4.1 Analysis of C4BP and B2GPI Interaction in the Presence of Protein S

C4BP interacted directly with B2GPI bound to oxidized ELISA plate but not with control wells coated with 5% milk. Interaction of C4BP with B2GPI was calcium-dependent as inclusion of 10 mM EDTA in the incubation reaction partially inhibited C4BP binding to B2GPI (p < 0.001, two way ANOVA) (Figure 14A). The presence of 50 µg/mL (930 nM) fluid phase B2GPI did not inhibit C4BP (0 to 10 µg/mL, 0 to 17 nM) binding to B2GPI adsorbed to oxidized ELISA plate (Figure 14B). This suggests that fluid phase B2GPI does not interact with C4BP and fluid phase B2GPI exhibits a conformation different from ELISA plate-bound B2GPI. This indicates that the conformational alteration B2GPI undergoes when bound to an oxidized surface is important for C4BP binding. Similar to the binding of protein S to C4BP, the binding of B2GPI to C4BP was also mediated through the β-chain as there was significantly less binding of C4BP.β to B2GPI when incubated together (p < 0.001, two way ANOVA).
Figure 14. Interaction Between β2GPI and C4BP.

(A) Interaction between β2GPI and C4BP in the presence of calcium. Microtitre wells coated with β2GPI were incubated with C4BP with 2.5 mM CaCl₂ (closed circles) or 10 mM EDTA (open circles). Data points are the means ± 1 SD of three separate measurements of the same sample. The data are representative of ≥3 separate experiments. The OD₄₅₀ reported represents the amount of C4BP binding after subtracting the OD₄₅₀ of control wells that did not contain C4BP. (B) Fluid Phase β2GPI Did Not Affect C4BP Binding to Oxidized ELISA Plate-Bound β2GPI. Microtitre wells coated with β2GPI were incubated with 0 to 10 μg/mL (0 to 17 nM) C4BP with (closed circles) or without 50 μg/mL (930 nM) β2GPI (open circles). Data points are the means ± 1 SD of three separate measurements of the same sample. The data are representative of 3 separate experiments. The OD₄₅₀ reported represents the amount of C4BP binding after subtracting the OD₄₅₀ of control wells that did not contain C4BP.
A

B

OD_{450}

C4BP (μg/mL)

OD_{450}

[C4BP] (μg/mL)

*p < 0.001
Therefore, B2GPI may play a role in preventing protein S from binding C4BP by competing for the β-chain.

Since both C4BP and protein S were found to bind to B2GPI, C4BP and protein S were co-incubated in B2GPI-coated wells to examine whether these two proteins were bound to the same binding site or different binding sites on B2GPI. C4BP (200 μg/mL, 340 nM) was able to inhibit protein S (0 to 10 μg/mL, 0 to 145 nM) binding to B2GPI (p < 0.001, two way ANOVA) (Figure 16A). Interestingly, C4BP (0 to 10 μg/mL, 0 to 17 nM) binding to B2GPI was not inhibited by protein S (20 μg/mL, 290 nM); therefore, two different binding sites are likely to be present (Figure 16B). These observations suggest the possibility that the binding of C4BP to the C4BP-binding site of B2GPI altered the protein S-binding site on B2GPI and thereby inhibited protein S binding. However, this protein S inhibition could also be due to protein S-C4BP complex formation during incubation. To examine whether this inhibition of protein S binding was solely due to C4BP binding to B2GPI, B2GPI-coated wells were pre-incubated with C4BP, washed and then incubated with protein S. This experiment was performed to determine whether the B2GPI-C4BP complex could have the exact same inhibitory effect on protein S binding. The results indicate that B2GPI bound-C4BP partially inhibited protein S binding to the B2GPI-C4BP complex (p < 0.001, two way ANOVA) (Figure 16A). This suggests that B2GPI bound-C4BP as well as C4BP-protien S complex formation both play a role in inhibiting protein S binding to B2GPI. Therefore, oxidized surface bound-B2GPI 1) prevented protein S from binding to C4BP by competing for the β-chain on C4BP, and 2) interacted directly with protein S when not occupied by C4BP.
Figure 15. Interaction Between β2GPI and C4BP is dependent on the β-subunit of C4BP. Microtitre wells coated with β2GPI were incubated with C4BP (closed circles), C4BP_β (open circles) with 2.5 mM CaCl₂. Wells coated with blocking solution only (crosses) controlled for non-specific binding. Data points are the means ± 1SD of three separate measurements of the same sample. The OD₄₅₀ reported represents the amount of C4BP or C4BP_β binding after subtracting the OD₄₅₀ of control wells that did not contain C4BP or C4BP_β, respectively.
Figure 16. Inhibition of Protein S Binding to β2GPI by C4BP.

Microtitre wells coated with β2GPI were incubated with: (A) 0 to 10 μg/mL (0 to 145 nM) protein S with (open circles) or without 200 μg/mL (340 nM) C4BP (closed circles) or incubated with 200 μg/mL (340 nM) C4BP for 2 hr prior to protein S addition (crosses). Data points are the mean ± 1 SD of three separate measurements of the same sample. Data are representative of ≥3 separate experiments. The OD_{450} reported represents the amount of protein S binding after subtracting the OD_{450} of control wells that did not contain protein S. The error bars are smaller than the symbols. (B) 0 to 10 μg/mL (0 to 17 nM) C4BP with (open circles) or without 20 μg/mL (290 nM) protein S (closed circles). Data are representative of ≥3 separate experiments. The OD_{450} reported represents the amount of C4BP binding after subtracting the OD_{450} of control wells that did not contain C4BP.
4.2 Equilibrium Binding Constants

From time course studies, the binding of C4BP to β2GPI reaches equilibrium after 60 minutes at 37°C (Appendix Figure 6). The equilibrium dissociation constant of C4BP binding to ELISA-plate immobilized β2GPI after incubating for 4 hours at 37°C was $3.46 \pm 0.74 \times 10^{-7}$M and a C4BP/β2GPI molar ratio of $1.0 \pm 0.1$. Binding assays with only labeled C4BP or mixtures of labeled and unlabeled C4BP of different ratios also gave comparable binding constants (Appendix Figure 8).

4.3 Effects of β2GPI-Depletion From Plasma Using Anti-β2GPI-Sepharose Beads on Functional Protein S Levels

It had been shown previously by our research group that depletion of plasma β2GPI using anti-β2GPI coupled to Sepharose beads could decrease the immunological free protein S level (Marjan, 1994). This occurred through the direct binding of plasma protein S to antibody-bound β2GPI. As a result, only 65% of immunological free protein S remained compared to control plasma treated with non-immunogenic rabbit IgG-Sepharose. In this experiment, the effect of β2GPI depletion by anti-β2GPI Sepharose on plasma protein S functional activity level (i.e. clotting time measurement) was examined. Functional protein S assays on anti-β2GPI-Sepharose treated plasma did not show any significant changes in protein S activity compared to control (Figure 17). These results were in contrast to the immunologic free protein S measurements. This suggested that the depletion of β2GPI would lead to lower immunological free protein S levels but unaltered functional protein S activity. The underlying reason for these
Figure 17. Functional protein S levels in β2GPI-depleted plasma.

In six separate experiments, plasma was incubated with anti-human-β2GPI-Sepharose (black bars) or normal IgG-Sepharose (white bars) then tested for functional protein S activity. Each sample was assayed in duplicate; the mean value is reported.
seemingly opposite observations may be due to a sensitivity of free protein S activity to changes in β2GPI concentration. The depletion of β2GPI may actually allow the residual protein S to bind anionic phospholipids in the functional assay more efficiently because of the lack of competition with β2GPI. As a result of the more efficient binding, protein S may exhibit a normal activity level even though the actual amount of protein S is decreased.

4.4 Effects of Sepharose-coupled β2GPI on Functional Protein S Level

The β2GPI-depletion studies described above suggested that β2GPI might modulate protein S activity in plasma and that the removal of β2GPI can affect protein S activity. To examine the specific effect of β2GPI on protein S activity without disturbing the β2GPI level, plasma was incubated with β2GPI-Sepharose. After the incubation, β2GPI-Sepharose was removed and the protein S activity in plasma was measured. This procedure allowed interaction of plasma protein S with exogenous β2GPI without affecting the final concentration of β2GPI. This experiment was repeated six times in duplicate (Figure 18). The protein S activity in samples incubated with β2GPI-Sepharose was significantly lower than that of normal IgG-Sepharose treated samples (p < 0.05 by paired t-test).

4.5 Summary

β2GPI interacted with protein S and C4BP. Analyses of the interactions among β2GPI, protein S, and C4BP showed that oxidized surface-bound β2GPI 1) prevented protein S from binding to C4BP by competing for the β-chain, and 2) interacted directly
Figure 18. Functional protein S levels in plasma treated with β2GPI-Sepharose.

Plasma was incubated with β2GPI-Sepharose (black bars), or normal IgG-Sepharose (white bars) then functional protein S activity was measured. Values from six separate experiments are shown. Each sample was assayed in duplicate; mean values are shown.
with protein S when β2GPI was not occupied by C4BP. Thus, β2GPI may have a role in maintaining free protein S level.
5.1 Characteristics and Specificities of IgG Pools

Plasma samples from 10 patients positive for aPL IgG (aPL) and with a history of thromboses were pooled. The characteristics of each plasma sample are summarized in Table 3. The levels of both anticardiolipin and anti-β2GPI antibodies were measured. Anticardiolipin antibodies were detected by cardiolipin-coated ELISA plate. This ELISA detects antibodies specific for β2GPI as well as other phospholipid-binding serum proteins because of the use of fetal calf serum as sample diluent. Anti-β2GPI antibodies were detected by β2GPI-coated oxidized ELISA plate. In contrast to the anticardiolipin ELISA, this ELISA detects only antibodies specific for β2GPI. Antibodies detected in patients with APS interact with a variety of phospholipid-binding proteins (Roubey, 1994); however, β2GPI is the dominant antigen of aPL. When the aPL IgG pool used in these experiments was tested for its ability to bind to β2GPI, APC, protein S or C4BP, only wells coated with β2GPI showed increased binding of aPL relative to NH IgG (Figure 19). Specific xenogeneic antisera readily detected the presence of the particular protein used to coat the well (data not shown). Therefore, the lack of reactivity of aPL with proteins other than β2GPI was not due to failure to coat the microtitre wells.
Table 3. Characteristics of aPL IgG Pool.

<table>
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<th>Patient Plasma</th>
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<th>Anti-β2GPI ELISA</th>
</tr>
</thead>
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<tr>
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<td>IgG (GPU)</td>
<td>IgM (MPU)</td>
</tr>
<tr>
<td>A</td>
<td>&gt; 150.0</td>
<td>&gt; 150.0</td>
</tr>
<tr>
<td>B</td>
<td>&gt; 150.0</td>
<td>5.5</td>
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<tr>
<td>C</td>
<td>&gt; 150.0</td>
<td>&gt; 150</td>
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<tr>
<td>D</td>
<td>109.8</td>
<td>&gt; 150.0</td>
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<td>E</td>
<td>&gt; 150.0</td>
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<td>H</td>
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<td>I</td>
<td>&gt; 150.0</td>
<td>2.2</td>
</tr>
<tr>
<td>J</td>
<td>113.4</td>
<td>19.5</td>
</tr>
</tbody>
</table>

GPU  IgG Phospholipid Unit
MPU  IgM Phospholipid Unit
SGU  Standard IgG anti-β2GPI Unit
SMU  Standard IgM anti-β2GPI Unit
Figure 19. Specificity of the aPL pool for β2GPI.

Microtitre plates coated with 10 μg/mL of β2GPI, APC, protein S, or C4BP were incubated with aPL IgG (closed) or NH IgG (open) and binding measured as described in Methods. Values represent specific IgG binding (buffer/secondary antibody binding controls subtracted) and are means ± 1 SD of three separate measurements of the same sample. The data shown are representative of 3 separate experiments.
5.2 Inhibition of APC, Protein S, and C4BP Binding to β2GPI by aPL IgG pool

Studies have shown that aPL react directly with neo-epitope(s) expressed on β2GPI when β2GPI is bound to oxidized ELISA plates. Whether this aPL binding would inhibit the binding of APC, protein S, and C4BP to ELISA plate-bound β2GPI was investigated. Antiphospholipid IgG pool (aPL IgG), but not normal human IgG (NHIgG), inhibited the interaction of bound β2GPI with fluid phase APC, protein S, and C4BP (p < 0.001, two way ANOVA) (Figure 20). This inhibition increased with the amount of aPL in a dose-dependent manner (p < 0.001, two way ANOVA) (Figure 21).

5.3 Effects of aPL on APC Activity as Measured by Chromogenic Substrate S-2366

In this experiment, the effect of aPL IgG on β2GPI potentiation of APC activity on a PS surface was evaluated. The activity of PS-bound APC was quantified by measuring the hydrolysis of S-2366, a chromogenic substrate of APC. As shown in Figure 22A, when APC was co-incubated with β2GPI and NHIgG, the specific activity of PS-bound APC was 48 OD₄₀₅/μg. In contrast, the specific activity of PS-bound APC incubated with β2GPI and aPL IgG was 76 OD₄₀₅/μg. Therefore, the presence of aPL increased APC specific activity by 58% (p < 0.001, two way ANOVA). The effect of protein S on APC specific activity under the same incubation conditions was also examined. PS-bound APC had a specific activity of 44 OD₄₀₅/μg when co-incubated with protein S, β2GPI and NHIgG, whereas the specific activity of PS-bound APC
Figure 20. Inhibition of APC, protein S, and C4BP binding to β2GPI by aPL.

Microtitre plates coated with β2GPI (10 μg/mL) were incubated with 0 to 10 μg/mL of APC (top panel), protein S (middle panel), or C4BP (bottom panel) in the presence of 0.5 mg/mL of aPL pool (closed circles) or NHIgG pool (open circles). Data points are the means ± 1 SD of four separate measurements of the same samples. The data shown are representative of 3 separate experiments. The OD\textsubscript{450} reported represents the amount of APC, protein S, or C4BP binding after subtracting the OD\textsubscript{450} of control wells that did not contain APC, protein S or C4BP, respectively.
Figure 21. Inhibition of APC, protein S, and C4BP binding to β2GPI by aPL.

Microtiter plates coated with β2GPI (10 μg/mL) were incubated with 5 μg/mL of APC (top panel), protein S (middle panel) or C4BP (bottom panel) in the presence of 0 to 1 mg/mL of aPL (closed circles) and NH IgG (open circles). Data points are means ± 1 SD of four separate measurements of the same sample. The data shown are representative of 3 separate experiments. The OD$_{450}$ reported represents the amount of APC, protein S, or C4BP binding after subtracting the OD$_{450}$ of control wells that did not contain APC, protein S or C4BP, respectively.
Figure 22. Activity PS-Bound APC in the Presence of aPL β2GPI and Protein S.
PS-coated wells were incubated with 1) $^{125}$I-trace labeled APC (2 μg/mL, 32 nM) + β2GPI (100 μg/mL, 185 nM) + NHlgG (1 mg/mL, 6.7 μM), 2) APC (2 μg/mL, 32 nM) + β2GPI (100 μg/mL, 185 nM) + aPL (1 mg/mL, 6.7 μM), 3) APC (2 μg/mL, 32 nM) + β2GPI (100 μg/mL, 185 nM) + protein S (10 μg/mL, 145 nM) + NHlgG (1 mg/mL, 6.7 μM), or 4) APC (2 μg/mL, 32 nM) + β2GPI (100 μg/mL, 185 nM) + protein S (10 μg/mL, 6.7 μM) + aPL (1 mg/mL, 6.7 μM). (A) The specific activity of APC bound to PS-wells was obtained from the total activity generated by the PS-bound APC. (B) The total activity was quantified by measuring the hydrolysis of chromogenic substrate S-2366 (1 mM) after 60 minutes of incubation at RT with PS-bound APC. (C) The amount of APC bound to PS-coated surface measured by gamma-radiation counting. Data points are mean ± 1 SD of five separate measurements. Data are representative of 3 separate experiments.
A

![Graph A: Specific Activity (OD405/µg APC Bound)](image)

- PS Wells + APC
- PS Wells + APC + β2GPI
- PS Wells + APC + NHlG
- PS Wells + APC + Protein S
- PS Wells + APC + Protein S

B

![Graph B: Total APC Activity (OD405)](image)

- PS Wells + APC
- PS Wells + Protein S
- PS Wells + Protein S

C

![Graph C: APC Bound (ng)](image)

- PS Wells + APC
- PS Wells + Protein S
- PS Wells + Protein S

94
incubated with protein S, β2GPI and aPL IgG was 68 OD<sub>405</sub>/μg, a 55% increase (p <0.001, two way ANOVA). Therefore, the addition of protein S affected neither the APC specific activity in the presence of β2GPI and NH IgG or aPL IgG significantly nor the aPL-potentiation of APC specific activity. However, this aPL-mediated increase in APC specific activity in the presence of β2GPI did not lead into an increase in total APC activity. As shown in Figure 22B, when APC, β2GPI, and NH IgG were co-incubated in PS-coated wells, the total activity generated by PS-bound APC was an OD<sub>405</sub> of 0.390. The same incubation with aPL had an OD<sub>405</sub> of 0.344, a 13% reduction (p <0.001, two way ANOVA). This inhibitory effect was even greater for incubations containing protein S. The co-incubation of APC β2GPI, protein S and NH IgG had a total APC activity of 0.426 compared to 0.316 of incubation with aPL IgG, a 26% decrease (p <0.001, two way ANOVA). This decrease in total APC activity was directly correlated to the decrease in APC binding to phospholipid surface in the presence of aPL (Figure 22C).

5.4 aPL Increases β2GPI Binding to Phospholipid Surface

To examine the possible causes of decreased APC binding to PS in the presence of aPL, the binding of β<sup>125</sup>I-β2GPI to PS-coated ELISA plate in the presence of aPL and NH IgG were examined. The binding of β2GPI to PS surface was somewhat enhanced by aPL IgG (p < 0.001, two way ANOVA) (Figure 23). This result suggests that the decrease of APC binding to PS plate in the presence of aPL was at least in part due to the increased deposition of β2GPI to PS.
Figure 23. aPL Increases β2GPI Binding to Phospholipid Surface.

PS-coated ELISA plates were incubated with $^{125}$I-β2GPI (0 to 10 μg/mL) in the presence of 500 μg/mL aPL IgG (closed circles) and NH IgG (open circles). After the incubation, wells were washed with ice-cold TBS-Ca four times. The amounts of APC bound were measured by gamma-radiation counting. Data points are mean ± 1 SEM of three separate measurements.

![Graph showing binding of β2GPI to phospholipid surface](image)
5.5 Binding of APC, Protein S, and C4BP to Activated Platelets in the Presence of aPL and β2GPI

As described above, β2GPI interacted with APC, protein S, and C4BP when β2GPI was bound to an oxidized surface. Also, modulation of protein S-C4BP complex formation in the fluid phase by β2GPI has been demonstrated (Merrill et al., 1999). Since aPL IgG could inhibit β2GPI interactions with APC, protein S, and C4BP, the ability of aPL IgG to interfere with the binding of these proteins to the physiologically relevant surface of activated platelets was evaluated using flow cytometry. Washed platelets were activated with calcium ionophore, and the activation state of platelets was followed by measuring the expression of platelet activation marker CD62P. APC (4 μg/mL, 64 nM), protein S (20 μg/mL, 290 nM), and C4BP (250 μg/mL, 424 nM) binding to platelets were platelet activation-dependent as resting platelets did not support protein binding. The binding of APC and protein S to activated washed platelets was not affected by the addition of aPL IgG (1 mg/mL, 6.6 μM) compared to NHIgG (1 mg/mL, 6.6 μM). Similarly, the binding of a mixture of protein S and C4BP to activated platelets was also not affected by aPL IgG (Table 4). These observations suggest that aPL IgG does not interfere with the binding of APC, protein S, and C4BP to the activated platelet surface in the presence of β2GPI. The results indicate that even though aPL IgG could interfere with β2GPI interacting with other proteins on an oxidized surface as well as in fluid phase, the activated platelet is not a significant target surface where aPL IgG may exert its thrombogenic effect. The signals detected were due to specific binding of primary and secondary antibodies to proteins bound to activated platelets as the detection
of proteins bound to activated platelets with non-immunogenic isotypic primary antibodies did not produce significant signals.

5.6 Binding of β2GPI to Activated Platelets in the Presence of aPL IgG

The binding of β2GPI to activated platelets was examined in this experiment to determine whether this is the basis of the lack of aPL IgG modulation of APC, protein S, and C4BP binding to activated platelets in the above experiment. If there is indeed no β2GPI binding to activated platelets, then aPL IgG will not be able to inhibit APC, protein S, and C4BP binding to the cells by inhibiting β2GPI from interacting with the other proteins. When calcium ionophore-activated washed platelets were incubated with 1) $^{125}$I-β2GPI (200 μg/mL), 2) $^{125}$I-β2GPI and NHIgG (1mg/mL), and 3) $^{125}$I-β2GPI and aPL (1mg/mL), no $^{125}$I-β2GPI binding to activated platelets was observed. The same results were obtained when repeated with platelets from three normal subjects. In contrast, when $^{125}$I-protein S (20 μg/mL) was incubated with platelets, 10.3 ± 0.8 % of the protein S added was bound to calcium ionophore-activated platelets while only 0.8 % of protein S was bound to resting platelets. Therefore, there was significant protein S binding to activated platelets ($p < 0.002$, paired t-test, n=3). These results together indicate that even though activated platelets with anionic phospholipid exposed on cell surface can support protein S binding, β2GPI binding to activated platelets surface does not take place at physiological ionic strength, pH, and calcium concentration.
Table 4. Binding of Activated Protein C, Protein S and C4BP to Platelets as Detected by Fluorescence Flow Cytometry is not Affected by the Presence of aPL.

Final concentrations of proteins in the reaction mixtures were 200 μg/mL (3.7 μM) β2GPI, 4 μg/mL (64 nM) APC, 20 μg/mL (290 nM) protein S, 250 μg/mL (424 nM) C4BP, and 1 mg/mL (6.6 μM) of antibody pool for aPL or NHIgG. Where indicated, platelets were activated with A23187. Data are expressed as the mean ± one SD of four separate samples.

<table>
<thead>
<tr>
<th>Platelets incubated with</th>
<th>Stained with</th>
<th>Activated platelets + aPL</th>
<th>Activated platelets + NHIgG</th>
<th>Resting platelets + NHIgG</th>
<th>Activated platelets + NHIgG (Isotypic control)</th>
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<tbody>
<tr>
<td>β2GPI, Protein S, APC, aPL/NHIgG</td>
<td>anti-CD62P</td>
<td>78.7 ± 3.0</td>
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<td>14.4 ±4.7</td>
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<td>anti-Protein S</td>
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<td>anti-Protein C</td>
<td>8.7 ± 3.4</td>
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<tr>
<td>β2GPI, Protein S, C4BP, aPL/NHIgG</td>
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<td>anti-Protein S</td>
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</table>
5.7 Summary

Antiphospholipid antibodies inhibited APC, protein S, and C4BP binding to oxidized surface bound β2GPI in a concentration-dependent manner. Also, aPL was found to increase APC specific activity in the presence of β2GPI on PS-coated surface. However, this increase in APC specific activity did not lead to an increase in APC total activity because aPL, at the same time, inhibited APC binding to PS by increasing β2GPI binding. As a result of this inhibition, there was an overall decrease in APC total activity. The presence of aPL did not interfere with the binding of APC, protein S, and C4BP to activated platelet surface. Furthermore, no significant β2GPI binding to activated platelets was found and aPL did not enhance β2GPI binding to activated platelets.
CHAPTER 6 INHIBITION OF C4b-BINDING PROTEIN (C4BP) AND MANNOSE BINDING LECTIN (MBL) BINDING TO β2GPI BY ANTIPHOSPHOLIPID ANTIBODIES

6.1 Effect of C4b on C4BP Binding to β2GPI

C4BP was shown to interact with oxidized-surface bound β2GPI in the experiments described above. This interaction was mediated by the β-chain on C4BP. The implication of this interaction on complement regulation is examined in this section. To further assess whether β2GPI-bound C4BP can regulate complement activation by binding to C4b, C4BP (0 to 8 μg/mL, 0 to 14 nM) was pre-incubated with C4b (50 μg/mL, 260 nM) before adding to β2GPI-coated wells. This was to investigate whether the occupation of C4b-binding domains on C4BP by C4b would inhibit C4BP binding to β2GPI. The pre-incubation of C4BP with C4b did not inhibit C4BP binding to β2GPI compared to C4BP binding to β2GPI in the absence of C4b (Figure 24). The lack of inhibition by C4b showed that the C4b-binding domains on C4BP were not involved in C4BP-β2GPI interaction and further suggested that the β-chain of C4BP may be the sole binding site for β2GPI. Therefore, β2GPI-bound C4BP should have its C4b binding activity retained for complement regulation. Hence, the inhibition of C4BP binding to β2GPI by aPL observed in previous experiments may interfere with complement regulation.
Figure 24. Effect of C4b on C4BP binding to β2GPI.

Microtiter plates coated with β2GPI were incubated with C4BP (0 to 8 μg/mL, 0 to 14 nM) in the presence (closed circles) and absence (open circles) of C4b (50 μg/mL, 260 nM). Data points are means ± 1 SD of four separate measurements of the same sample. The data shown are representative of 3 separate experiments. The OD$_{450}$ reported represents the amount of C4BP binding after subtracting the OD$_{450}$ of control wells that did not contain C4BP.
6.2 MBL Binding to β2GPI in the Presence of Calcium, EDTA and N-acetylmannosamine

Using ELISA, in the presence of 2.5 mM CaCl₂, MBL was found to interact directly with surface-bound β2GPI in a concentration-dependent manner but not with control wells coated with skim milk protein (p < 0.001, two way ANOVA) (Figure 25A). In the presence of 10 mM EDTA, MBL binding to β2GPI was inhibited. Since the carbohydrate binding activity of MBL is calcium-dependent, the observation of EDTA inhibition of MBL-β2GPI interaction suggests that this interaction could be mediated through the binding of MBL to the carbohydrate residues on β2GPI. This possible mechanism was also examined using N-acetylmannosamine, which binds to MBL and inhibits its lectin activity. Galactose, which is not recognized by MBL, was used as control. MBL binding to β2GPI was inhibited by the addition of N-acetylmannosamine but not galactose (p < 0.001, two way ANOVA) (Figure 25B). Therefore, MBL binding to β2GPI is likely to be mediated by the binding of MBL to the sugar residues on β2GPI.

6.3 Binding of MBL to β2GPI-Bound aPL

In the earlier experiments, aPL was shown to prevent APC, protein S, and C4BP from binding to β2GPI. In this experiment, the inhibitory effect of aPL on MBL binding to β2GPI was examined. Contrary to the aPL inhibitory effect on APC, protein S, and C4BP, aPL increased MBL binding to β2GPI. When MBL (0 to 10 μg/mL, 0 to 20 nM) was co-incubated with 1 mg/mL (6.7 μM) of aPL IgG pool (> 150 GPU), there was significantly higher binding of MBL to β2GPI-coated wells than with 1 mg/mL (6.7 μM)
Figure 25. MBL Binding to β2GPI.

(A) Effect of EDTA on MBL binding. Microtitre wells coated with β2GPI were incubated with MBL (0 to 10 µg/mL, 0 to 20 nM) in 2.5 mM CaCl₂ (closed circles) or 10 mM EDTA (open circles). Wells coated with blocking solution only (crosses) controlled for non-specific binding. The OD₄₅₀ reported represents the amount MBL binding after subtracting the OD₄₅₀ of control wells that did not contain MBL.

(B) Effect of N-acetylmannosamine on MBL Binding. Microtitre plates coated with β2GPI were incubated with MBL (0 to 10 µg/mL, 0 to 20 nM) in the presence of 0.2 M N-acetylmannosamine (closed circles) or galactose (open circles). Data points are the means ± 1 SD of three separate measurements of the same sample. The data are representative of 3 separate experiments. The OD₄₅₀ reported represents the amount MBL binding after subtracting the OD₄₅₀ of control wells that did not contain MBL.
of NH IgG pool control (p < 0.001, two way ANOVA) (Figure 26). This indicates that MBL has higher affinity towards the aPL-β2GPI complex than towards β2GPI alone. Therefore, the presence of aPL may promote complement activation and enhance opsonization by co-localizing MBL with aPL-β2GPI complex.

6.4 Equilibrium Dissociation Constant Determinations for MBL Binding to β2GPI

To determine the equilibrium dissociation constants for MBL binding to β2GPI adsorbed to an ELISA plate, the time required for the protein binding to reach equilibrium was determined. Binding of 10 μg/mL (20 nM) of 125I-trace labeled-MBL to β2GPI was found to reach equilibrium after 60 min (Appendix Figure 7). The amount of MBL binding to surface-bound β2GPI interaction was determined by incubating β2GPI-coated wells with 125I-trace labeled MBL (0 to 80 μg/mL, 0 to 160 nM) for 4 hours at 37°C. The MBL-β2GPI interaction has a K_d of 7.43 ± 4.42x 10^{-7} M and a MBL:β2GPI molar ratio of 0.6 ± 0.3. The binding of 125I-labeled BSA to β2GPI was measured as control. There was a negligible interaction and the association of the two proteins was too weak for equilibrium dissociation constant determination using this experimental setup. Binding assays with only labeled MBL or mixtures of labeled and unlabeled MBL of different ratios also gave comparable binding constants (Appendix Figure 8).
Figure 26. Increased Binding of MBL to β2GPI in the Presence of aPL IgG.

Microtitre plates coated with β2GPI were incubated with MBL (0 to 10 μg/mL, 0 to 20 nM) in the presence of 1 mg/mL (6.6 μM) of aPL IgG pool (closed circles) or NHlgG pool (open circles). Data points are the means ± 1 SD of three separate measurements of the same samples. The data shown are representative of 3 separate experiments. The OD₄₅₀ reported represents the amount MBL binding after subtracting the OD₄₅₀ of control wells that did not contain MBL.
6.5 Complement Activation by Antibody-β2GPI Complex

To measure antibody-mediated complement activation, namely aPL-β2GPI complex-mediated complement activation, a complement activation ELISA was developed. Since rabbit IgG activates human classical complement pathway and has been routinely used to assay complement activity in human serum (Kent and Fife, 1963), rabbit-anti-human-β2GPI was initially used to develop this assay. After rabbit anti-human β2GPI was added to β2GPI-coated wells to form rabbit-anti-β2GPI/β2GPI complex, the amount of C5b-9 deposition in ELISA wells when incubated with human serum increased with the amount of rabbit anti-human β2GPI added initially (Figure 27). Therefore, the amount of complement activation measured was dependent on the amount of rabbit-anti-β2GPI/β2GPI complex under the experimental condition used.

Wells containing rabbit-anti-β2GPI/β2GPI complexes generated no signals when probed with non-specific mouse IgG as primary antibody or without primary antibody after incubating with serum. This indicated that the signals generated were not due to non-specific binding of primary antibody (mouse-anti-C5b-9) or secondary antibody (HRP-anti-mouse-IgG) to the ELISA plate. When rabbit-anti-β2GPI/β2GPI complexes were incubated with serum in GVB-EDTA and probed with anti-C5b-9 and the appropriate secondary antibody, no signal was produced either (data not shown). Since EDTA in GVB-EDTA buffer inhibits complement activation, this observation indicated that the signal generation was complement activation-dependent. Therefore, these controls together showed that signals produced were specific binding of anti-C5b-9 to C5b-9 bound to ELISA plate as a result of complement activation.
Figure 27. Rabbit Anti-Human β2GPI Mediated C5b-9 Generation.

β2GPI-coated ELISA plates were incubated with different amounts of rabbit-anti-human β2GPI in GVB-EDTA for 1 hr at room temperature. NHS diluted 40 times in GVB$^{2+}$ were added to the wells and incubated at 37°C for 1 hr for complement activation. The amount of C5b-9 generated after the incubation was measured using monoclonal mouse anti-human C5b-9. Data points are the means ± 1 SD of four separate measurements of the same samples. The data shown are representative of 3 separate experiments.
6.6  **β2GPI-Mediated Complement Activation**

The observation of direct MBL-β2GPI interaction suggests that β2GPI-coated surface should be complement activating in the presence of serum by activating MBL. In Figure 28, β2GPI-coated wells incubated with serum had significantly higher amounts of complement activation (OD$_{450}$ of 0.117 ± 0.017, n = 6) compared to gelatin-blocked control wells (OD$_{450}$ of 0.080 ± 0.005, n = 5) (p < 0.001 by t-test). Therefore, β2GPI-coated surface may be complement activating, albeit weak in comparison to the complement activating activity of antibody-β2GPI complex observed in the above experiment.

6.7  **Correlations of Complement Activation Level to Antiphospholipid Levels**

Using the complement activation ELISA, aPL-mediated complement activation by patient serum was assessed. Samples with complement activation levels more than OD$_{450}$ of 0.257 (mean + 3 SD, 10 normal serum samples) were considered as positive. Antiphospholipid antibody mediated complement activation was detected in 19 (47.5 %) patient samples (Table 5). Serum samples positive for complement activation were selected to determine the correlations of complement activation levels to aCL IgG, aCL IgM, anti-β2GPI IgG, and anti-β2GPI IgM levels. Of the 19 samples positive for aPL-mediated complement activation, a correlation coefficient of 0.45 was found between the level of complement activation and the level of anti-β2GPI IgM (p<0.05). No correlation was established between complement activation and the levels of aCL IgG, aCL IgM, or anti-β2GPI IgG (Table 6). Therefore, anti-β2GPI IgM may be the main
Figure 28. \( \beta 2\text{GPI-Mediated Complement Activation.} \)

NHS diluted 1 in 40 with \( \text{GVB}^2+ \) were added to \( \beta 2\text{GPI-coated} \) (\( n=6 \)) and gelatin-coated (\( n=5 \)) ELISA wells and incubated at 37\(^\circ\)C for 1hr for complement activation. The amount of C5b-9 generated after the incubation was measured using monoclonal mouse anti- human C5b-9. Data points are the means ± 1 SD. The data shown are representative of 3 separate experiments.
Table 5. Characteristics of Serum Samples.

Ten normal and 40 patient samples were analyzed using complement activation ELISA, anticardiolipin ELISA, and anti-B2GPI ELISA. Sample is positive if complement activation > 0.257, GPU > 15, MPU >10, SGU >15, or SMU > 15. Samples positive for any of these ELISAs is shaded grey in the table.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Complement Activation (OD&lt;sub&gt;450&lt;/sub&gt;)</th>
<th>Anti-Carciolipin ELISA</th>
<th>Anti-B2GPI ELISA</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>IgG (GPU)</td>
<td>IgM (MPU)</td>
<td>IgG (SGU)</td>
</tr>
<tr>
<td>Normal 1</td>
<td>0.201</td>
<td>2.5</td>
<td>0.4</td>
</tr>
<tr>
<td>Normal 2</td>
<td>0.231</td>
<td>1.6</td>
<td>2.9</td>
</tr>
<tr>
<td>Normal 3</td>
<td>0.212</td>
<td>3.3</td>
<td>2.3</td>
</tr>
<tr>
<td>Normal 4</td>
<td>0.182</td>
<td>3.6</td>
<td>1.8</td>
</tr>
<tr>
<td>Normal 5</td>
<td>0.210</td>
<td>6.3</td>
<td>1.5</td>
</tr>
<tr>
<td>Normal 6</td>
<td>0.157</td>
<td>4.0</td>
<td>1.7</td>
</tr>
<tr>
<td>Normal 7</td>
<td>0.198</td>
<td>4.0</td>
<td>10.0</td>
</tr>
<tr>
<td>Normal 8</td>
<td>0.189</td>
<td>2.8</td>
<td>0.9</td>
</tr>
<tr>
<td>Normal 9</td>
<td>0.189</td>
<td>4.3</td>
<td>1.4</td>
</tr>
<tr>
<td>Normal 10</td>
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<td>8.9</td>
<td>10.0</td>
</tr>
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<td>1.2</td>
</tr>
<tr>
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<td>14.0</td>
</tr>
<tr>
<td>Patient 3</td>
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<td>39.8</td>
<td>0.6</td>
</tr>
<tr>
<td>Patient 4</td>
<td>0.198</td>
<td>&gt; 150.0</td>
<td>&gt; 150.0</td>
</tr>
<tr>
<td>Patient 5</td>
<td>0.189</td>
<td>34.0</td>
<td>1.5</td>
</tr>
<tr>
<td>Patient 6</td>
<td>0.205</td>
<td>13.6</td>
<td>9.6</td>
</tr>
<tr>
<td>Patient 7</td>
<td>0.195</td>
<td>2.3</td>
<td>20.5</td>
</tr>
<tr>
<td>Patient 8</td>
<td>0.213</td>
<td>25.3</td>
<td>&gt; 150.0</td>
</tr>
<tr>
<td>Patient 9</td>
<td>0.199</td>
<td>44.2</td>
<td>0.0</td>
</tr>
<tr>
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<td>0.202</td>
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<td>6.2</td>
</tr>
<tr>
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<td>0.422</td>
<td>8.9</td>
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</tr>
<tr>
<td>Patient 12</td>
<td>0.208</td>
<td>25.0</td>
<td>0.9</td>
</tr>
<tr>
<td>Patient 13</td>
<td>0.325</td>
<td>24.6</td>
<td>5.5</td>
</tr>
<tr>
<td>Patient 14</td>
<td>0.279</td>
<td>6.6</td>
<td>1.5</td>
</tr>
<tr>
<td>Patient 15</td>
<td>0.279</td>
<td>&gt; 150.0</td>
<td>5.5</td>
</tr>
<tr>
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<td>8.3</td>
<td>5.6</td>
</tr>
<tr>
<td>Patient 17</td>
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<td>&gt; 150.0</td>
<td>&gt; 150</td>
</tr>
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<td>0.206</td>
<td>12.9</td>
<td>16.9</td>
</tr>
<tr>
<td>Patient 19</td>
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<td>3.0</td>
</tr>
<tr>
<td>Patient 20</td>
<td>0.574</td>
<td>32.9</td>
<td>&gt; 150.0</td>
</tr>
<tr>
<td>Patient 21</td>
<td>1.473</td>
<td>109.8</td>
<td>&gt; 150.0</td>
</tr>
<tr>
<td>Patient 22</td>
<td>1.335</td>
<td>49.2</td>
<td>1.0</td>
</tr>
<tr>
<td>Patient 23</td>
<td>0.726</td>
<td>69.1</td>
<td>2.3</td>
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<td>Patient 24</td>
<td>0.861</td>
<td>17.1</td>
<td>1.6</td>
</tr>
<tr>
<td>Patient 25</td>
<td>0.150</td>
<td>&gt; 150.0</td>
<td>10.9</td>
</tr>
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<td>Patient 26</td>
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<td>58.9</td>
<td>19.1</td>
</tr>
<tr>
<td>Patient 27</td>
<td>0.177</td>
<td>16.4</td>
<td>9.6</td>
</tr>
<tr>
<td>Patient 28</td>
<td>0.906</td>
<td>32.9</td>
<td>&gt; 150.0</td>
</tr>
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<td>Patient 29</td>
<td>0.150</td>
<td>150</td>
<td>28.6</td>
</tr>
<tr>
<td>Patient 30</td>
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<td>37.4</td>
<td>9.7</td>
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<tr>
<td>Patient 31</td>
<td>0.154</td>
<td>18.4</td>
<td>1.9</td>
</tr>
<tr>
<td>Patient 32</td>
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<td>59.5</td>
<td>&gt; 150.0</td>
</tr>
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<td>Patient 33</td>
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<td>&gt; 150.0</td>
<td>4.3</td>
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<td>0.265</td>
<td>44.2</td>
<td>6.6</td>
</tr>
<tr>
<td>Patient 35</td>
<td>0.210</td>
<td>&gt; 150.0</td>
<td>3.6</td>
</tr>
<tr>
<td>Patient 36</td>
<td>0.243</td>
<td>19.7</td>
<td>43.5</td>
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<td>Patient 37</td>
<td>0.154</td>
<td>5.8</td>
<td>&gt; 150.0</td>
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<tr>
<td>Patient 38</td>
<td>0.301</td>
<td>&gt; 150.0</td>
<td>2.2</td>
</tr>
<tr>
<td>Patient 39</td>
<td>0.246</td>
<td>150</td>
<td>0.0</td>
</tr>
<tr>
<td>Patient 40</td>
<td>0.246</td>
<td>113.4</td>
<td>19.5</td>
</tr>
</tbody>
</table>

Patients Positive | 19 | 31 | 19 | 21 | 24 |

(47.5 %) (77.5 %) (47.5%) (52.5 %) (60.0 %)
Table 6. Correlation (r) of Complement Activation to Antiphospholipid Level.

Nineteen of the forty-six serum samples positive for complement activation were selected to determine the correlation of complement activation levels to antiphospholipid levels.

<table>
<thead>
<tr>
<th>Antigen</th>
<th>Correlation of Complement Activation to Antiphospholipid Level</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti-cardiolipin IgG</td>
<td>0.14</td>
</tr>
<tr>
<td>Anti-cardiolipin IgM</td>
<td>0.25</td>
</tr>
<tr>
<td>Anti-β2GPI IgG</td>
<td>0.11</td>
</tr>
<tr>
<td>Anti-β2GPI IgM</td>
<td>0.45*</td>
</tr>
</tbody>
</table>

*p<0.05
complement activator in antiphospholipid syndrome. However, anti-β2GPI IgM is not a mandatory activator of complement since patients positive for anti-β2GPI IgM (patients 7, 8, 9, 10, 11, 18, 26, 27, 30, 31, 33, 37, 40) did not exhibit aPL-β2GPI complex mediated complement activation. The observation of significant complement activation for patients 3, 20, 23 who were negative for antiphospholipid IgM, further showed that besides IgM, antiphospholipid of IgG isotype could also support complement activation (Table 5).

6.8 Specificity and Sensitivity of Antiphospholipid ELISAs Relative to Complement Activation ELISA

The application of antiphospholipid ELISAs (aCL IgG, aCL IgM, anti-β2GPI IgM, and anti-β2GPI IgG) in identifying APS patient samples containing complement activating antiphospholipid antibodies was examined (Table 7A-D). The anticardiolipin IgG ELISA had a sensitivity of 78.9% relative to the complement activation ELISA while the other three ELISAs only had moderate sensitivities (42.1 to 57.9%). The relatively high sensitivity of anticardiolipin IgG ELISA indicates that complement activation is associated with the presence of anticardiolipin antibodies. The specificities of these other ELISAs relative to the complement activation ELISA were moderate (23.8 to 47.6%). The low specificities indicate that not all antiphospholipid antibodies activate complement. Examination of the positive predictive values of the different antiphospholipid ELISAs relative to complement activation ELISA also only showed moderate values (38.1 to 48.4%). Therefore, none of these antiphospholipid ELISAs by themselves would be able to predict complement activation.
Table 7. Specificity and Sensitivity of Antiphospholipid ELISAs Relative to Complement Activation ELISA.

<table>
<thead>
<tr>
<th></th>
<th>Anti-cardiolipin IgG</th>
<th>Anti-cardiolipin IgM</th>
<th>Anti-β2GPI IgG</th>
<th>Anti-β2GPI IgM</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>+ 15 16</td>
<td>- 4 5</td>
<td>+ 8 13</td>
<td>- 11 10</td>
</tr>
<tr>
<td>Specificity</td>
<td>= 23.8 %</td>
<td></td>
<td>= 38.1 %</td>
<td>= 38.1 %</td>
</tr>
<tr>
<td>Sensitivity</td>
<td>= 78.9 %</td>
<td></td>
<td>= 42.1 %</td>
<td>= 57.9 %</td>
</tr>
<tr>
<td>Positive Predictive Value</td>
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<td>= 38.1 %</td>
<td>= 45.8 %</td>
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<tr>
<td>Negative Predictive Value</td>
<td>= 55.6 %</td>
<td></td>
<td>= 42.1 %</td>
<td>= 50.0 %</td>
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</table>
6.9 Summary

β2GPI interacted with C4BP and MBL. Antiphospholipid antibodies inhibited C4BP binding while enhancing MBL binding to oxidized surface bound β2GPI. These modulation effects of aPL on β2GPI could generate a condition that promotes complement activation. Nineteen out of forty (47.5%) patients serum samples led to the generation of C5b-9 in complement activation ELISA. A moderate correlation was found between the level of C5b-9 produced and the level of anti-β2GPI IgM. No correlations were found between the level of C5b-9 produced and the levels of anticardiolipin IgG/IgM and anti-β2GPI IgG in the sera.
CHAPTER 7 DISCUSSION

In this study, a number of interactions of phospholipid-bound or oxidized surface-bound β2GPI with the anticoagulation mechanism as well as with the complement system have been demonstrated. β2GPI interacted with APC and had different effects on APC under different conditions. Also, β2GPI appeared to modulate the formation of C4BP-protein S complex by interacting directly with both C4BP and protein S. Furthermore, β2GPI was associated with the complement system by interacting with C4BP and MBL. Experiments have shown that these interactions were interfered with by the presence of aPL. These observations suggest that in the antiphospholipid syndrome, aPL may interfere with these protein interactions, and thereby disrupt the anticoagulation mechanism and increase complement activation.

7.1 β2GPI Interactions with APC and Protein S

The anticoagulation activity of APC can be impaired by aPL (Galli et al., 1998; Oosting et al., 1993; Malia et al., 1990; Marciniak and Romond, 1989). However, the mechanism that mediates this inhibition and the role of β2GPI in this phenomenon remains unclear. Therefore, the interaction between APC and β2GPI was explored to gain an understanding of aPL-mediated APC inhibition. The effect of β2GPI on APC activity in the presence of aminophospholipid (PE and PS), PC, protein S as well as in the absence of phospholipid was studied. Experiments showed that β2GPI could serve as an APC cofactor to increase APC specific activity (Figure 29). Since both proteins are
Figure 29. β2GPI Increases the Specific Activity of APC in the Presence of Aminophospholipid.
phospholipid-binding proteins, this increase in specific activity may result from complex formation between APC and β2GPI subsequent to phospholipid binding. This increase in APC specific activity in the presence of β2GPI, however, did not translate into increased total APC activity for the *in vitro* assay. This may be because β2GPI, similarly to APC, is an anionic phospholipid binding protein. On a PS surface, β2GPI competed with APC for PS binding and because less APC bound, less total activity was observed. Therefore, compared to APC incubation in a PS-well alone, co-incubation of APC and β2GPI in a PS-well led to a lowered APC total activity because of the decrease of APC binding to PS, even though β2GPI increased APC specific activity. The concentrations of protein used in this experiment were chosen to approximate the physiological protein concentration ratios of β2GPI and APC in plasma with the assumption that all protein C (4 µg/mL, 64 nM) (Griffin *et al.*, 1982) is activated to become APC. Whether these proteins interact *in vivo* at this ratio or with different local concentration ratios is unknown. This point is important because for maximum APC potentiation, β2GPI and APC should interact at an optimum ratio where all phospholipid-bound β2GPI would be interacting with APC. If there were an excess of β2GPI compared to APC, β2GPI would actually reduce total APC activity by competing with APC for phospholipid sites rather than increase APC specific activity by interacting with the protein. Therefore, the *in vivo* function of β2GPI would depend on the balance of β2GPI-mediated APC enhanced activity and the inhibition of APC binding by β2GPI to a procoagulant surface. This phenomenon should be considered when interpreting *in vitro* experiments that examine the effect of β2GPI on APC activity in the presence of phospholipid since APC activity
would be directly affected by the ratios of the amounts of any of these components chosen for the experiments.

APC inactivates membrane bound FVa by binding in close proximity to FVa on the same procoagulant surface containing anionic phospholipids such as PS (Kalafatis and Mann, 1993; Mesters et al., 1993; Freyssinet et al., 1991; Walker, 1981). PE, which has a neutral net charge at physiological pH, has also been reported to be an important phospholipid for APC binding (Smirnov et al., 1995; Horie et al., 1994; Smirnov and Esmon, 1994). Analysis of APC specific activity when it was bound to different phospholipids showed that APC bound to PC had the highest specific activity, followed by PE and PS. Furthermore, similar to PS-bound APC, the specific activity of PE-bound APC was also increased in the presence of β2GPI. In contrast, the specific activity of PC-bound APC was unchanged. This suggests that β2GPI has PE-binding activity that enhances APC activity. This PE-binding activity of β2GPI has previously been reported using a liposomal model (Harper et al., 1998). The lack of enhancement of APC activity in PC-coated wells is probably due to the negligible binding of β2GPI to PC. These observations together suggest β2GPI may serve to enhance APC activity on surfaces containing aminophospholipids. Even though PC-bound APC exhibited higher specific activity than PE-bound and PS-bound APC, PC-bound APC probably is a minor or even negligible contributor to APC anticoagulation activity by itself. This is because APC bound only weakly to PC but strongly to PE and PS. Consequently, higher total APC activity was observed in PE- and PS- coated wells even though PE-bound and PS-bound APC had a lower specific activity. Therefore, the total activity of APC exhibit on a phospholipid surface should be a function of both the amount of APC deposited and the
specific activity of APC on the phospholipid. Thus, a mixture of phospholipid that allows strong APC binding and high APC specific activity should give the highest total APC activity. In fact, this may be one of the factors for the observation that phospholipid vesicles containing 20% PS and 80% PC are the optimum phospholipid mixture to support APC-mediated FVa inactivation whereas vesicles with either higher or lower percent of PS sustained only lower APC activity (Bakker et al., 1992). Also, the fact that the Gla-domain contains a combination of anionic phospholipid binding region as well as hydrophobic binding site (Jalbert et al., 1996; Christiansen et al., 1995; Zhang and Castellino, 1994) further raises the possibility that APC may exhibit higher enzymatic activity when a mixture of different phospholipids is available for the Gla-domain.

APC binding to β2GPI was found to be independent of aminophospholipids as APC readily bound to β2GPI adsorbed to oxidized ELISA plates in a calcium-dependent manner. Therefore, the aminophospholipids may serve to localize the two proteins in close proximity for interaction. Studies have shown that when β2GPI is adsorbed to the oxidized polymer of an ELISA plate, it will undergo conformational changes and express neo-epitopes for β2GPI-dependent antiphospholipid autoantibody binding (Chamley et al., 1999; Ichikawa et al., 1994; Matsuura et al., 1994; Roubey, 1994; Kandiah and Krilis, 1994; Arvieux et al., 1991). The importance of this β2GPI conformational alteration for APC binding was examined using a competitive ELISA. The presence of fluid phase β2GPI did not affect APC binding to solid phase β2GPI. This observation suggests that the conformational alteration that β2GPI undergoes upon adsorption to an oxidized surface is critical for β2GPI interaction with APC and that the interactions between oxidized surface-bound β2GPI and fluid phase APC are much stronger than the
fluid phase interaction between B2GPI and APC if the latter occurs at all. Protein S also interacted with oxidized surface-bound B2GPI in a very similar manner. Protein S-B2GPI interaction was calcium-dependent and specific for the conformation expressed by B2GPI when oxidized-surface bound.

APC function was also assessed in this system to determine whether oxidized surface-bound B2GPI has the same potentiating effect on APC specific activity as phospholipid-bound B2GPI. APC activity was inhibited by oxidized surface-bound B2GPI. Therefore, B2GPI bound to an oxidized surface expressed an effect on APC opposite to B2GPI bound to phospholipids. This inhibition could be due to the suboptimal orientation of APC for S-2366 hydrolysis when bound to B2GPI adsorbed to ELISA plate without phospholipid. However, it is possible that B2GPI expresses different conformations depending on the binding surface. If this is correct, B2GPI may serve to potentiate as well as inhibit APC activity depending whether it is in the APC potentiation conformation or the APC inhibition conformation. This possibility may have an important implication in the way that anti-B2GPI autoantibodies are detected in plasma samples. Currently, two different ELISAs are used in detecting anti-B2GPI autoantibodies. One has B2GPI directly coated to oxidized ELISA plate. The other one has negatively charged phospholipid, usually cardiolipin, coated to the ELISA plate and uses fetal calf serum as a source of B2GPI. The two methods for assessment of B2GPI-dependent aPL may induce different B2GPI conformational changes and therefore, capture different subsets of conformation-specific aPL.

Since both APC and protein S can interact with B2GPI, APC and protein S were co-incubated in B2GPI-coated wells to examine whether they could interfere with the
binding of each other to β2GPI. When 10 μg/mL (145 nM) of protein S was incubated with 10 μg/mL (160 nM) of APC or less, APC binding to β2GPI was significantly inhibited. Therefore, it is likely that protein S has a role in inhibiting APC binding to β2GPI since APC exists at an even lower concentration in plasma than in this experiment. The concentration of APC in circulating blood is around 2 ng/mL (32 pM) (Gruber and Griffin, 1992). The local concentration of APC at a site of coagulation activation would be predicted to have a higher APC concentration, however, the APC concentration would still be substantially lower than the protein S concentration at the coagulating surface because of the lower plasma concentration of its precursor, protein C (4 μg/mL, 64 nM) (Griffin et al., 1982) compared to protein S (20 μg/mL, 290 nM) (Schwarz et al., 1986; Fair and Revak, 1984; Dahlback, 1983b). Also, APC has a lower affinity for negatively charged phospholipid (K\text{d} of 1.5 \times 10^{-7} \text{ M}) exposed at the injured site than protein S (K\text{d} of 5 \times 10^{-8} \text{ M}) (Walker, 1981). In contrast, when 10 μg/mL (160 nM) of APC were incubated with 10 μg/mL (145 nM) of protein S or less, protein S binding to β2GPI was unaffected. Therefore, in vivo, APC is unlikely to affect protein S binding to β2GPI since the APC concentration would not attain such high levels relative to protein S in plasma as in the experiment. These observations suggest that APC and protein S may bind to different binding sites on β2GPI where the occupation of the protein S binding site by protein S would inhibit APC interaction with the APC binding site by either steric hinderance or conformational alteration of APC binding site. This inhibition of β2GPI-APC interaction by protein S was studied using oxidized plate-bound β2GPI. As discussed above, oxidized surface-bound β2GPI may express a conformation different from phospholipid-bound β2GPI. Therefore, phospholipid-bound β2GPI may not be
similarly affected by protein S. In fact, using PS-coated wells, the addition of protein S to APC and β2GPI incubation showed neither inhibition of β2GPI-meidated potentiation of APC nor additive or synergistic potentiation of APC. This suggests that protein S has no effect on β2GPI-APC interaction when β2GPI is phospholipid bound. Thus β2GPI may express a protein S-binding conformation only when adsorbed to oxidized surface but not when bound to phospholipid. Therefore, protein S may serve to prevent β2GPI on an oxidized surface from inhibiting APC where β2GPI is in the APC inhibition conformation but not when β2GPI is in the APC potentiation conformation on a phospholipid surface.

Protein C, the precursor of APC, also interacted with β2GPI. The equilibrium dissociation constants for APC and protein C binding ELISA plate-bound β2GPI were both estimated to be 800 to 900 nM based on the assumption that all β2GPI adsorbed to the plate were in the optimum orientation for APC and protein C binding. Therefore, the actual equilibrium dissociation constants would be expected to be even lower depending on how optimally β2GPI was oriented for interaction. The plasma concentrations of β2GPI and protein C are 3.7 μM and 70 nM respectively. Therefore, the binding constant predicts a notable amount of interaction between protein C and β2GPI. This is also true for APC and β2GPI interactions assuming APC is present at a local concentration similar to the protein C plasma concentration during anticoagulation. In vivo, thrombomodulin-thrombin complex activates protein C to generate APC (Esmon and Owen, 1981). Whether the protein C-β2GPI interaction is also involved in this reaction remains to be determined. Protein S interacted with β2GPI with a molecular ratio of 2.9 ± 0.4 (protein S/β2GPI). Since β2GPI is a modular protein consisting of five SCR domains, this protein
S-β2GPI molecular ratio could have resulted from interactions of more than one protein S molecule with different SCR domains on the same β2GPI molecule. The binding of $1.5 \pm 0.2$ APC molecules to one β2GPI was probably the result of APC-β2GPI interaction in a similar manner, namely an averaging of the combination of 2:1 and 1:1 binding. An analogous observation of two distinct binding sites for C3b and one C4b binding site on complement receptor 1, a protein with 30 SCR domains, has been reported (Klickstein et al., 1988). Also, the binding of C4b (Hardig et al., 1997, Kristensen et al., 1987, Dahlback et al., 1983) and factor I (Chung and Reid, 1985) to different SCR domains on C4BP further demonstrate the interactions of proteins with different SCRs on the same molecule.

In summary, β2GPI can potentiate APC specific activity in the presence of aminophospholipid, namely PE and PS. Aminophospholipids are normally sequestered to the inner leaflet of the phospholipid bilayer of platelets as well as other cells and only expressed when cells are activated or lysed. Therefore, the potentiation of APC activity by β2GPI probably occurs as a coordinated event associated with the concomitant event of aminophospholipid expression during platelet activation or apoptosis. The perturbation of this β2GPI-APC interaction by β2GPI-binding antiphospholipid antibodies may explain impaired APC activity and increased risk of thrombosis observed in patients with antiphospholipid syndrome. However, the association of β2GPI deficiency and thrombotic risk has yet to be established. Even though β2GPI deficiency was initially reported not to be a risk factor for thrombosis (Bancsi et al., 1992), there is now growing evidence that the level of β2GPI may be linked with thrombotic risk.
(Brighton et al., 1996; McNally et al., 1995; McNally et al., 1995; Vlachoyiannopoulos et al., 1992).

7.2 β2GPI Interactions with Protein S and C4BP

β2GPI has been shown to down-regulate protein S-C4BP complex formation (Merrill et al., 1999; Atsumi et al., 1997). The mechanism of this tri-molecular interaction was further characterized and confirmed here by investigating the interactions of the protein S-C4BP system with β2GPI bound to an oxidized surface or to Sepharose beads in both a purified protein system and plasma. Results indicate that β2GPI can interact with C4BP in a calcium-dependent manner. Also, β2GPI can prevent the formation of the protein S-C4BP complex by competing with protein S for the β-chain of C4BP. As a result of this competition, β2GPI may function to increase free protein S level by promoting the dissociation of protein S from protein S-C4BP complex.

Conformational alteration of β2GPI by adsorbing to an oxidized surface was an important pre-requisite for this interaction since fluid phase β2GPI did not inhibit C4BP from binding to β2GPI adsorbed to the plate. This indicates that the conformationally altered β2GPI adsorbed to oxidized surfaces can bind to fluid phase C4BP more readily than fluid phase β2GPI does. Taken together, these findings suggest that oxidized surface bound-β2GPI 1) prevented protein S from binding to C4BP by competing for the β-chain on C4BP, and 2) interacted directly with protein S when β2GPI was not occupied by C4BP (Figure 30). These results however are in discrepancy with the report by Merrill et al (Merrill et al., 1999) that showed β2GPI in fluid phase can inhibit protein S binding to
C4BP. This discordance perhaps demonstrates that β2GPI can modulate protein S-C4BP complex formation in two different ways. In fluid phase, β2GPI maintains the free protein S level by controlling protein S binding to C4BP systemically. When a suitable surface or biological process alters the β2GPI conformation, the dissociation of protein S from C4BP becomes much more rapid and leads to a dramatic increase in free protein S local concentration. Surfaces that may allow β2GPI binding include activated platelets (Vazquez-Mellado et al., 1994; Nimpf et al., 1985; Schousboe, 1983), endothelial cells (Meroni et al., 1998; George et al., 1998; Del Papa et al., 1998; Del Papa et al., 1997; Del Papa et al., 1997; Del Papa et al., 1995; Le Tonqueze et al., 1995), lipoproteins (Gambino et al., 1999; Cardigan et al., 1998; Hasunuma et al., 1997; McNally et al., 1996; Polz et al., 1981; Polz et al., 1980), apoptotic bodies (Levine et al., 1998; Balasubramanian and Schroit, 1998; Manfredi et al., 1998; Balasubramanian et al., 1997; Price et al., 1996), as well as certain strains of Staphylococcus aureus (Zhang et al., 1999).

Exposure of plasma to anti-β2GPI Sepharose also decreased the immunologically measurable free protein S levels compared to controls, another indication of direct β2GPI-protein S interaction (Marjan, 1994). However, functional protein S assays of anti-β2GPI-Sepharose treated plasma did not show any significant changes in protein S activity compared to control. These results were in contrast to the immunologic free protein S measurements. This suggested that the depletion of β2GPI would lead to lower immunological free protein S level but unaffected functional protein S activity. The underlying reason for these seemingly opposite observations may be due to a sensitivity of free protein S activity to changes in β2GPI concentration. The depletion of β2GPI
Figure 30. β2GPI Interactions with C4BP and Protein S.
may actually allow the residual protein S to bind anionic phospholipids in the functional assay more efficiently because of the lack of competition with β2GPI. As a result of more efficient binding, protein S would exhibit a normal activity level even though the actual amount of protein S was decreased. Therefore, β2GPI may modulate protein S activity in plasma and the removal of β2GPI could affect protein S activity. The specific effect of β2GPI on protein S activity was then examined by incubating plasma with β2GPI-Sepharose. After the incubation, β2GPI-Sepharose was removed and protein S activity in plasma was measured without disturbing the β2GPI level. Functional protein S assays of these samples showed that plasma incubated with β2GPI-Sepharose had lower protein S activity levels; thus the level of β2GPI may modulate free protein S activity in such assays. These observations suggest that β2GPI, protein S, and anionic phospholipid together are intricately balanced in the anticoagulation mechanism. Also, the fact that protein S interacted with β2GPI-Sepharose suggests that the crosslinking of β2GPI to Sepharose may also induces conformational alterations on β2GPI similar to those caused by the adsorption to an oxidized surface.

The experiments in this study demonstrated that conformationally altered β2GPI was able to prevent protein S-C4BP complex formation as well as interact with protein S. However, whether β2GPI-bound protein S still possesses anticoagulant activity upon binding remains to be determined. This interaction could be procoagulant by causing the local reduction in protein S concentration in the presence of conformationally altered β2GPI with a concomitant loss of free protein S cofactor activity. Alternatively, β2GPI-protein S interaction might form a complex that is anticoagulant. The latter scenario
seems more likely considering the observation of increased protein S activity when exogenous β2GPI was added (Merrill et al., 1999).

7.3 Effects of aPL on β2GPI Interactions with APC, Protein S, and C4BP

Strong associations between the presence in a patient’s blood of antibodies with reactivity to β2GPI on an ELISA plate and thrombosis have been reported (Day et al., 1998; Arnout et al., 1998; Takeya et al., 1997; Swadzba et al., 1997; Willems et al., 1996; Pengo et al., 1996; Cabiedes et al., 1995). Studies have indicated that strong binding of aPL to β2GPI is mediated by the expression of neo-epitope(s) on β2GPI when bound to oxidized surface (Ichikawa et al., 1994; Matsuura et al., 1994; Roubey, 1994; Kandiah and Krilis, 1994; Arvieux et al., 1991). Experiments have shown here that an aPL IgG pool could inhibit APC, protein S, and C4BP binding to oxidized surface bound β2GPI in a concentration-dependent manner. Since APC and protein S are important physiologic anticoagulants, the interference of the normal interactions of β2GPI with these hemostatic proteins may lead to thrombogenesis.

Investigations of the thrombogenic mechanisms involved in APS suggested that APC activities could be impaired by antiphospholipid antibodies (aPL). Sixty-two percent of 42 APS patients in the study of Galli et al. (Galli et al., 1998), 23% of 30 APS patients in the study of Oosting et al. (Oosting et al., 1993), 78% of 9 APS patients in the study of Malia et al. (Malia et al., 1990), and 100% of 15 patients positive for LA in the study of Marciniak et al. (Marciniak and Romond, 1989) exhibited inhibition of APC-mediated FVa inactivation. In another study, 47% of 78 aPL positive patients antibodies
bound protein C via β2GPI (Atsumi et al., 1998). Therefore, inhibition of APC activity is a prevalent feature for APS patients. A reduction in total APC activity in the presence of pooled aPL IgG was also observed using the APC activity ELISA developed for this study. Despite the decrease in total APC activity, a concomitant increase in APC specific activity associated with the addition of by aPL IgG in the presence of β2GPI was observed. This increase in APC specific activity, however, did not lead to a higher total APC activity. This is because aPL IgG inhibited APC binding to the PS-coated wells at same time. As a result of this inhibition, there was an overall decrease in APC total activity. Therefore, compared to APC incubation in PS-well co-incubated with β2GPI and NHIGG, co-incubation of APC, β2GPI and aPL IgG in PS-well led to a lower APC total activity because of the decrease of APC binding to PS, even though the presence of aPL increased APC specific activity. This enhancement of APC specific activity was probably mediated by the increase in β2GPI surface density on phospholipid in the presence of aPL. When β2GPI was incubated with aPL, more β2GPI was bound to the PS-coated surface, an effect which has also been demonstrated previously by different groups using different experimental conditions (Arnout et al., 1998; Takeya et al., 1997; Willems et al., 1996). The result of the increased surface density of β2GPI on PS-coated surface may be to promote efficient interaction between β2GPI and APC and, consequently, increase APC specific activity. However, this interpretation is contradicted by the observation that aPL could block APC from interacting with β2GPI bound to an oxidized surface in this study. This seemingly contradictory information may originate from the heterogeneity of antiphospholipid antibodies. Studies of monoclonal aPL IgG generated from APS patients showed that patients possessed intra- and inter-individual
heterogeneity of antiphospholipid antibodies and that different clones of antibodies are specific for β2GPI in different conformations or bound to different surfaces (Zhu et al., 1999; Shan et al., 1998). For example, some monoclonal antibodies are specific for β2GPI bound to either anionic phospholipid or an oxidized ELISA plate while some monoclonal antibodies are only specific for β2GPI bound to one type of surface or the other. As a result, it is possible that the antibodies that blocked APC from binding to β2GPI bound to oxidized surface would loss the inhibitory effect when β2GPI is bound to phospholipid. Therefore, to gain clear understanding of the relationship between aPL specificity and thrombogenic mechanism involved, analyses of the specificities of clones of autoantibodies individually from patients would be essential. The need to use monoclonal aPL to dissect the complex pathogenic mechanism of these autoantibodies had been discussed by Zhu et al (Zhu et al., 1999). Antiphospholipid antibodies of different isotypes, subclasses, and specificities have been generated by different groups (Olee et al., 1996; Harmer et al., 1995; Ichikawa et al., 1994; Hasegawa et al., 1994). These antibodies will be indispensable in the elucidation of the pathogenic mechanism by allowing the identification clones of aPL that are specific for domains on β2GPI critical for the maintenance of hemostatic balance.

Antiphospholipid antibody binding to activated platelets in the presence of β2GPI had been detected using flow cytometry (Reverter et al., 1995). Radiolabeled anti-β2GPI aPL binding to activated platelets in the presence of β2GPI was observed (Shi et al., 1993). Direct β2GPI binding and β2GPI-mediated aPL binding to activated platelets were also reported using flow cytometry and FITC-labeled β2GPI and aPL (Vazquez-Mellado et al., 1994). Furthermore, anti-β2GPI with lupus anticoagulant activity was
demonstrated to inhibit prothrombinase activity on activated platelet surface (Galli et al., 1993). These observations together strongly suggest that activated platelets can support β2GPI-mediated anti-β2GPI autoantibodies deposition on activated platelet surface and modulation of coagulation activity as a result of this binding. Since aPL could inhibit β2GPI interactions with APC, protein S, and C4BP, the ability of aPL to interfere with the binding of these proteins to the surface of activated platelets using flow cytometry in the presence of β2GPI was tested. However, the presence of aPL did not interfere with APC, protein S, and C4BP binding to activated platelets. This result suggests that even though aPL could interfere with β2GPI interacting with other proteins on oxidized surface in the above experiments as well as in fluid phase (Merrill et al., 1999), activated platelets are not the target surface where aPL exert its thrombogenic effect.

Recent studies have suggested that β2GPI does not bind significantly to activated platelets (Brighton et al., 1999; Harper et al., 1998). Platelet binding experiments confirmed studies reporting that even though activated platelets with anionic phospholipid exposed on cell surface can support protein S binding, β2GPI does not bind significantly to activated platelets at physiological ionic strength, pH, and calcium concentration (2.5 mM Ca$^{2+}$). Also, aPL had no effect on β2GPI binding to activated platelets. This is in discordance with studies that showed aPL and β2GPI binding to activated platelets (Reverter et al., 1995; Vazquez-Mellado et al., 1994; Galli et al., 1993; Shi et al., 1993). This discrepancy may originate from variations in the final concentration of calcium ion used in the incubation mixtures. β2GPI binding to anionic phospholipid is very sensitive to the presence of calcium ions. Using different techniques, marked inhibition of binding was observed at physiologic calcium
concentration compared to the absence of calcium (Wang et al., 1999; Harper et al., 1998; Nimpf et al., 1985; Wurm, 1984). In studies that showed aPL and β2GPI binding to activated platelets (Reverter et al., 1995; Vazquez-Mellado et al., 1994; Shi et al., 1993), calcium was not included in the incubation mixture. This may allow increased β2GPI binding to activated platelets. A recent study further underlined the importance of calcium on the activity of anti-β2GPI lupus anticoagulant (Pengo et al., 1999). In this study, anti-β2GPI lupus anticoagulants were shown to exhibit lupus anticoagulant activity in the dRVVT clotting assay only when plasma and antibodies were incubated with phospholipid before calcium ions was added as the clotting initiator. When phospholipid was incubated with calcium prior being exposed to plasma and antibodies, anti-β2GPI was not able to exhibit LA activity. The loss of anti-β2GPI lupus anticoagulant activity in the dRVVT clotting assay by a simple alteration of incubation sequence of calcium showed that calcium is an important modulator of the in vitro anticoagulant activity of anti-β2GPI autoantibodies. This effect is likely to be caused by calcium inhibition of β2GPI binding to phospholipid. When calcium is not present, β2GPI can bind to phospholipid in the presence of anti-β2GPI unimpeded, whereas the presence of calcium would inhibit β2GPI binding. Since β2GPI could not bind to PL in the presence of calcium, anti-β2GPI would not be able to exert LA activity on PL surface. These observations suggest that β2GPI binding as well as β2GPI-mediated anti-β2GPI binding to activated platelets would also be strongly affected by calcium. Thus, anti-β2GPI and β2GPI may not be able to bind to activated platelets at the physiological concentration of calcium. Besides the concentration of calcium, other factors may also contribute the difference in β2GPI and aPL binding to activated platelets in different studies. One of the
major factors is the activity of the aPL used in the various studies. Different amounts of total aPL activities were used and this made comparison of the different studies difficult. Therefore, to better evaluate the binding of β2GPI and aPL to activated platelets, the calcium concentration and the activity of aPL required to induce in vitro anticoagulant activity or in vivo procoagulant activity need to be re-examined with these findings in mind.

7.4 Effects of aPL on β2GPI Interactions with Complement System and MBL

Elevated levels of complement activation (Hammond et al., 1989) and hypocomplementemia have been reported for patients with APS (Arvieux et al., 1991; Hazeltine et al., 1988). Therefore, the complement activation process in APS and the possible role of β2GPI were examined to gain more understanding of the underlying mechanism. Experiments showed that C4BP could interact with oxidized surface-bound β2GPI through the β-chain of C4BP and that the C4b-binding sites of C4BP were not involved in C4BP-β2GPI interaction. From these observations, the C4b-binding sites on β2GPI-bound C4BP appeared to be unoccupied and available for complement regulation. Thus, β2GPI may play a role in localizing C4BP to surfaces that permit β2GPI binding. However, this interaction can be inhibited by aPL. Perturbation of this interaction by aPL can potentially dysregulate complement regulation and lead to the elevated complement activation in APS.

Mannose binding lectin (MBL) is an oligomeric protein involved in the classical complement pathway activation (Ohta et al., 1990; Lu et al., 1990; Ikeda et al., 1987).
Purification of B2GPI using anti-human B2GPI immunoaffinity Sepharose beads led to the co-immunoprecipitation of MBL (Marjan, 1994). Thus, it is possible that there is a specific interaction between MBL and B2GPI. Using ELISAs, direct MBL-B2GPI interaction was found to proceed in a calcium-dependent fashion. Binding studies have shown that the calcium-dependent carbohydrate recognition domain of MBL binds only to sugar containing an equatorial 4-OH group (e.g. N-acetylmannosamine, mannose, N-acetylglucosamine and glucose) but not sugar containing an axial 4-OH (e.g. N-acetylgalactosamine and galactose) (Elgavish and Shaanan, 1997; Kawasaki et al., 1989).

Since MBL-B2GPI interaction was inhibited by N-acetylmannosamine but not galactose in the experiments performed here, the interaction was most likely mediated by the carbohydrate recognition domains on MBL and the sugar residues on B2GPI. Carbohydrate analyses of B2GPI from other studies indeed show that B2GPI contains sugar residues that can interact with MBL, namely terminal mannose and terminal fucose residues (Gambino et al., 1997; Gambino et al., 1997; Gambino et al., 1997). MBL-B2GPI interaction had a $K_d$ of $7.43 \pm 4.42 \times 10^{-7}$ M and a MBL/B2GPI molar ratio $0.6 \pm 0.3$ at $37^\circ$C. The dissociation constant for MBL binding to a strain of E. coli rich in mannose-derivatives on the bacteria surface is $6 \times 10^{-9}$ M (Kawasaki et al., 1989).

Therefore, MBL binding to E. coli is two orders of magnitude stronger than binding to B2GPI. However, MBL also has phospholipid binding activity (Kilpatrick, 1998). The stronger affinity observed for MBL binding to sugar residues on E. coli than binding to sugar residues on purified B2GPI may result from a combined effect of MBL binding to sugar residues together with MBL binding to phospholipids of E. coli.
In the presence of aPL, an increased amount of MBL deposition to β2GPI-coated surface was observed. This may have implications in elevated complement activation and enhanced opsonization on MBL-aPL-β2GPI coated surfaces, such as activated platelets. It has been shown that when MBL is deposited on a cell surface, it can act as an opsonin to facilitate the phagocytosis and clearance of the coated particles (Tenner et al., 1995; Malhotra et al., 1990). Different mechanisms may lead to the increase in MBL binding to aPL-β2GPI complex. Antiphospholipid antibodies may have induced conformational changes on β2GPI for MBL binding to the β2GPI sugar residues more efficiently or more avidly. Also, it is possible that the β2GPI-bound aPL contributes its sugar residues, together with the sugar residues on β2GPI for MBL to bind more avidly to the aPL-β2GPI complex. Autoantibodies of the IgG isotype developed in rheumatoid arthritis (rheumatoid factors) have been shown to undergo glycosylation changes to unmask the terminal N-acetylgalactosamine residue in the Fc region for MBL binding (Malhotra et al., 1995). As a result, these IgGs can activate the classical complement pathway by MBL through the lectin pathway (Sato et al., 1997). Whether the mechanism involved in the elevated MBL binding to aPL-β2GPI complex is mediated by the glycosylation changes on aPL similar to the autoantibodies in rheumatoid arthritis remains to be further investigated.

Associations between MBL deficiency and recurrent fetal wastage have been observed in different studies. The deficiency of MBL in maternal serum is associated with unexplained recurrent miscarriage (Christiansen et al., 1999; Kilpatrick et al., 1995) and pre-eclampsia (Kilpatrick, 1996). Recurrent fetal wastage and pre-eclampsia (Alsulyman et al., 1996; Lima et al., 1996; Kon et al., 1995) are also common
manifestations in antiphospholipid syndrome. Therefore, the possibility of aPL-β2GPI complex causing recurrent miscarriage by sequestering MBL from blood and inducing MBL-deficiency deserves further attention (Malhotra et al., 1994). The observation of aPL-mediated enhanced MBL binding to β2GPI may possibly have a causal relationship with pregnancy complications in APS.

Using the complement activation ELISA, sera containing populations of antiphospholipid antibodies that could activate complement by forming aPL-β2GPI complexes were identified. The activation of complement and formation of C5b-9 may lead to platelet activation and thrombosis, as C5b-9 is an effective platelet activator (Sims et al., 1988). Analysis of serum samples positive for complement activation ELISA revealed a moderate correlation of anti-β2GPI IgM titer to complement activation. However, a correlation of anti-β2GPI IgG titer to complement activation could not be established. A lack of correlation between aPL titer and the aPL-mediated C5b-9 deposition on cardiolipin-coated polystyrene beads has also been previously been observed using flow cytometry (Stewart et al., 1997). That report suggested that this could be caused by the difference in avidity of aPL immunoglobulin in the different samples. As a result, samples with aPL immunoglobulins of high avidity would exhibit strong complement activation whereas samples with aPL immunoglobulins of low avidity would exhibit weak complement activation. The observation of the binding of C4BP to β2GPI suggests it is also possible that the level of C4BP in patients’ serum samples could affect the relationship between aPL titer and complement activation. When serum samples were incubated in β2GPI-coated wells, aPL and C4BP would bind to the wells concomitantly. Therefore, it is possible to have weak complement activation even for a
high aPL titer sample if the amount of β2GPI-bound C4BP is high. Also, the lack of correlation may be caused by differences in the relative amount of the different IgG subclass in aPL IgG. Among the four IgG subclasses, the complement C1q activating activity of the different subclasses is in the following order: IgG3 (40x), IgG1 (6x), IgG2 (1x) and IgG4 (undetectable) (Hamilton, 1987). Therefore, the extent of complement activation by aPL in serum sample will depend on not only the aPL level, but the relative amounts of IgG3, IgG1 and IgG2 to IgG4 of aPL. Studies have shown that aPL occurs in all four subclasses with the weak complement activator IgG2 subclass (80%) as the most prevalent type (Sammaritano et al., 1997; Arvieux et al., 1994; Levy et al., 1990; Gharavi et al., 1988). One of these studies also showed that in about 25% of the patients positive for both anticardiolipin and anti-β2GPI, aPL IgG subclass was skewed towards IgG3 (30 to 60% of total anticardiolipin IgG) (Arvieux et al., 1994). Therefore, it is possible that a sample with high aPL titer would generate low complement activation if IgG2 is the predominant IgG subclass while a low aPL titer sample would generate high complement activation if the aPL IgG subclass distribution was skewed towards strong complement activating subclasses IgG3. This skewing of aPL IgG subclass may also explain the lack of correlation of aPL IgG titer and complement activation.

The application of antiphospholipid ELISAs (aCL IgG, aCL IgM, anti-β2GPI IgM, and anti-β2GPI IgG) in determining which APS samples will contain these complement activating antiphospholipid antibodies was ineffective. None of the ELISAs was a strong predictor of the presence of complement activating aPL. This indicates that the complement activating population of antibodies is not restricted to anticardiolipin or anti-β2GPI. Furthermore, the complement activating property of antiphospholipid was
neither confined to IgM nor IgG isotype. In contrast, positivity in the complement activation ELISA was strongly predictive of the presence of antiphospholipid antibodies. Therefore, only some of the APS patients will have subsets of aPL that induce complement activation.

In summary, β2GPI may play a unique role in the complement system by interacting with C4BP and MBL simultaneously to balance the inhibition and the activation of complement on β2GPI-enriched surface. The presence of aPL may disrupt this balance and promote complement activation and C5b-9 formation. Complement activation on the platelet surface in the presence of aPL as a result of C5b-9 deposition has been demonstrated (Stewart et al., 1997).

7.5 Future Directions

In this study, β2GPI was found to modulate APC activity and regulate protein S-C4BP complex formation. One of the major findings is that β2GPI can enhance anticoagulation by increasing APC specific activity as well as inhibit anticoagulation by decreasing APC binding to phospholipid. Antiphospholipid antibodies also exhibited this dual effect on APC. However, the precise conditions under which one effect would be dominant over the other are unknown. The determination of conditions under which these two competing effects would be in equilibrium would give insight into the condition that would change an antithrombotic environment into a prothrombotic one. Also, the characterization of β2GPI-mediated APC potentiation with the physiological substrate FVa would yield insight into the enzymatic mechanism involved.
Phospholipid-bound and oxidized ELISA plate-bound β2GPI exhibited different effects on APC and protein S. This is probably due to the different conformation β2GPI expressed when bound to different surfaces. Structural studies could be undertaken to gain understanding of the interrelationships between different conformations and β2GPI activities. Monoclonal anti-β2GPI antibodies can also be used to map epitopes that are expressed only after conformational alteration. This may help in identifying domains on β2GPI important for modulating APC and protein S activity as well as pinpointing regions where thrombogenic aPL might bind. Also, the conformational changes of β2GPI when bound to different surfaces, such as endothelial cells, apoptotic bodies, lipoproteins, and S. aureus, would provide knowledge about the role of β2GPI on these surfaces.
CHAPTER 8 SUMMARY

Patients with APS are predisposed to recurrent thrombosis, thrombocytopenia, and recurrent miscarriages. Elevated levels of complement activation and hypocomplementernemia have also been reported in patients with APS. The adsorption of β2GPI to an oxidized surface is necessary to express neo-epitope(s) for aPL binding (Chamley et al., 1999; Ichikawa et al., 1994; Matsuura et al., 1994; Roubey, 1994; Kandiah and Krilis, 1994; Arvieux et al., 1991). Strong associations between thrombosis and aPL binding to β2GPI ELISA also exist (Day et al., 1998; Swadzba et al., 1997; Pengo et al., 1996; Cabiedes et al., 1995). Therefore, the possible interactions among neo-epitope(s) expressed by β2GPI and APC, protein S, C4BP, and MBL were investigated.

β2GPI interacted with two anticoagulation proteins, APC and protein S. β2GPI potentiated APC specific activity on the aminophospholipids PE and PS, but not PC. This enhancement of APC specific activity indicated that there could be a direct interaction between β2GPI and APC. This direct interaction was confirmed to be independent of aminophospholipid as APC could bind directly to β2GPI adsorbed to oxidized ELISA plate (K₅ 8.7 x 10⁻⁷ M). Therefore, the aminophospholipids served only to localize the two proteins in close proximity for interaction. In addition, APC binding to oxidized surface-bound β2GPI was not inhibited by fluid phase β2GPI. This suggested that oxidized surface-bound β2GPI expressed a conformation different from the fluid phase β2GPI to support APC binding. Further investigation revealed that protein S could inhibit APC binding to β2GPI adsorbed to an oxidized surface. Therefore, protein S may
be involved in the regulation of the potentiation of APC specific activity by β2GPI on
aminophospholipids.

β2GPI also interacted with protein S (K_d 7.35 x 10^{-7} M) as well as C4BP (K_d 3.46
x 10^{-7} M), a complement regulator. C4BP and protein S circulate as a complex in blood.
Analyses of the interactions between β2GPI, protein S, and C4BP showed that oxidized
surface-bound β2GPI 1) prevented protein S from binding to C4BP by competing for the
β-chain, and 2) interacted directly with protein S when β2GPI was not occupied by
C4BP. Thus, β2GPI may have a role in maintaining free protein S level (Figure 31).

APC inhibition is a prevalent feature of patients with APS. However, the
mechanism involved has not been established. Antiphospholipid antibodies inhibited
APC, protein S, and C4BP binding to oxidized surface bound β2GPI in a concentration-
dependent manner. Also, aPL was found to increase APC specific activity in the
presence of β2GPI on PS-coated surface. However, this increase in APC specific activity
did not lead to an increase in APC total activity because aPL, at the same time, inhibited
APC binding to PS by increasing β2GPI binding. As a result of this inhibition, there was
an overall decrease in APC total activity (Figure 32). Using flow cytometry, the effect of
aPL on APC, protein S, and C4BP binding to activated platelets in the presence of β2GPI
was examined. The presence of aPL did not interfere with the binding of these proteins
to activated platelet surface. Furthermore, no significant β2GPI binding to activated
platelets was found and aPL did not enhance β2GPI binding to activated platelets. These
observations together indicated that even though aPL can inhibit APC activity, aPL does
not exert its thrombogenic effect through activated platelets.
Figure 31. Effects of aPL on β2GPI interaction with protein S-C4BP complex.

(A) In the absence of aPL, β2GPI may function to prevent protein S from binding to C4BP by competing for the β-chain, and to interact directly with protein S when β2GPI is not occupied by C4BP. Thus, β2GPI may have a role in maintaining free protein S level.

(B) In the presence of aPL, β2GPI may be inhibited from interacting with protein S-C4BP complex by aPL. As a result, β2GPI would not increase free protein S level.

(A) **In the absence of aPL**

(B) **In the presence of aPL**
Figure 32. Effects of aPL on APC Activity.

(A) β2GPI may normally function to potentiate APC anticoagulation activity by forming β2GPI-APC complex. (B) The presence of aPL would inhibit APC binding by increasing β2GPI deposition on the anionic phospholipid and lead to lower total APC activity.
Besides C4BP, β2GPI also interacted with another complement protein, MBL ($K_d = 7.43 \times 10^{-7}$ M). MBL is a complement activator. Antiphospholipid antibodies inhibited C4BP binding while enhancing MBL binding to oxidized surface bound β2GPI. These modulation effects of aPL on β2GPI could generate a condition that promotes complement activation. β2GPI-coated ELISA wells were used to capture aPL from patient serums to form aPL-β2GPI complexes. These aPL-β2GPI complexes were incubated with normal human serum and the levels of complement activation were quantified by measuring C5b-9 formation. Nineteen out of forty (47.5 %) patients serum samples contained aPL that could lead to the generation of C5b-9. A moderate correlation was found between the level of C5b-9 produced and the level of anti-β2GPI IgM. No correlations were found between the level of C5b-9 produced and the levels of anticardiolipin IgG/IgM and anti-β2GPI IgG in the sera. However, C5b-9 formation was only observed in samples containing aPL. The interactions among β2GPI, C4BP, MBL, and aPL may have significance for clinical manifestations of APS such as complement activation, platelet activation, thrombocytopenia, and recurrent pregnancy loss.

In summary, β2GPI may normally interact with APC, protein S, C4BP, and MBL to maintain anticoagulation and regulate complement activation. However, in APS patients, aPL may interfere with these protein interactions, and thereby disrupt the anticoagulation mechanism, predisposing APS patients to thromboses, and increase complement activation.
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APPENDIX

Appendix Figure 1. Purity of β2GPI, APC, protein S, C4BP, C4BP\textsubscript{β}, and MBL. Proteins were analyzed using SDS-PAGE against molecular weight standards. Arrows indicate the position of the corresponding proteins. In the case of C4BP, the upper arrow indicates the position of the α-chain whereas the lower arrow indicates the position of the β-chain.
Appendix Figure 2. Effects of APC Iodination on APC Activity in PS-Coated Wells. PS-coated wells were incubated with 0 to 250 ng/mL of $^{125}$I-labeled APC (closed circles) or unlabeled APC (open circles). The activity of PS-bound APC was quantified by measuring the hydrolysis of chromogenic substrate S-2366 (1 mM) after 60 minutes of incubation at RT.
Appendix Figure 3. Dissociation Constant Determination for APC-β2GPI Interaction. Microtiter wells were coated with 100 μL of $^{125}$I-trace labeled β2GPI (10 μg/mL) in TBS overnight at 4°C and blocked with TBS-CaM. After washing with TBS-Ca, 100 μL of $^{125}$I-trace labeled APC (10 μg/mL) in TBS-Ca were incubated at 37°C. The amount of APC was quantified by gamma-radiation counting (top panel). The equilibrium dissociation constant for APC-β2GPI interaction was determined by incubating $^{125}$I-trace labeled APC (0 - 80 μg/mL) in wells previously coated with 100 μL $^{125}$I-trace labeled β2GPI (10 μg/mL) for 4 hours at 37°C. Proteins were removed and wells were washed with ice-cold TBS-Ca four times. The amounts of protein bound were measured by gamma-radiation counting. Binding constants were determined by non-linear curve fitting (bottom panel).
Appendix Figure 4. Dissociation Constant Determination for Protein C-β2GPI Interaction. Microtiter wells were coated with 100 μL of ¹²⁵I-trace labeled β2GPI (10 μg/mL) in TBS overnight at 4°C and blocked with TBS-CaM. After washing with TBS-Ca, 100 μL of ¹²⁵I-trace labeled protein C (10 μg/mL) in TBS-Ca were incubated at 37°C. The amount of protein C was quantified by gamma-radiation counting (top panel). The equilibrium dissociation constant for protein C-β2GPI interaction was determined by incubating ¹²⁵I-trace labeled protein C (0 - 80 μg/mL) in wells previously coated with 100 μL ¹²⁵I-trace labeled β2GPI (10 μg/mL) for 4 hours at 37°C. Proteins were removed and wells were washed with ice-cold TBS-Ca four times. The amounts of protein bound were measured by gamma-radiation counting. Binding constants were determined by non-linear curve fitting (bottom panel).
Appendix Figure 5. Dissociation Constant Determination for Protein S-β2GPI Interaction. Microtiter wells were coated with 100 μL of 125I-trace labeled β2GPI (10 μg/mL) in TBS overnight at 4°C and blocked with TBS-CaM. After washing with TBS-Ca, 100 μL of 125I-trace labeled protein S (10 μg/mL) in TBS-Ca were incubated at 37°C. The amount of protein S was quantified by gamma-radiation counting (top panel). The equilibrium dissociation constant for protein S-β2GPI interaction was determined by incubating 125I-trace labeled protein S (0 - 80 μg/mL) in wells previously coated with 100 μL 125I-trace labeled β2GPI (10 μg/mL) for 4 hours at 37°C. Proteins were removed and wells were washed with ice-cold TBS-Ca four times. The amounts of protein bound were measured by gamma-radiation counting. Binding constants were determined by non-linear curve fitting (bottom panel).
Appendix Figure 6. Dissociation Constant Determination for C4BP-B2GPI Interaction. Microtiter wells were coated with 100 µL of $^{125}$I-trace labeled β2GPI (10 µg/mL) in TBS overnight at 4°C and blocked with TBS-CaM. After washing with TBS-Ca, 100 µL of $^{125}$I-trace labeled C4BP (10 µg/mL) in TBS-Ca were incubated at 37°C. The amount of C4BP was quantified by gamma-radiation counting (top panel). The equilibrium dissociation constant for C4BP-β2GPI interaction was determined by incubating $^{125}$I-trace labeled C4BP (0 - 800 µg/mL) in wells previously coated with 100 µL $^{125}$I-trace labeled β2GPI (10 µg/mL) for 4 hours at 37°C. Proteins were removed and wells were washed with ice-cold TBS-Ca four times. The amounts of protein bound were measured by gamma-radiation counting. Binding constants were determined by non-linear curve fitting (bottom panel).
Appendix Figure 7. Dissociation Constant Determination for MBL-β2GPI Interaction.

Microtiter wells were coated with 100 µL of $^{125}$I-trace labeled β2GPI (10 µg/mL) in TBS overnight at 4°C and blocked with TBS-CaM. After washing with TBS-Ca, 100 µL of $^{125}$I-trace labeled MBL (10 µg/mL) in TBS-Ca were incubated at 37°C. The amount of MBL was quantified by gamma-radiation counting (top panel). The equilibrium dissociation constant for MBL-β2GPI interaction was determined by incubating $^{125}$I-trace labeled MBL (0 - 80 µg/mL) in wells previously coated with 100 µL $^{125}$I-trace labeled β2GPI (10 µg/mL) for 4 hours at 37°C. Proteins were removed and wells were washed with ice-cold TBS-Ca four times. The amounts of protein bound were measured by gamma-radiation counting. Binding constants were determined by non-linear curve fitting (bottom panel).
Appendix Figure 8. Effects of Iodination on APC-, protein C-, protein S-, C4BP-, and MBL- Binding to β2GPI.

Micrrotiter wells were coated with 100 μL of β2GPI (10 μg/mL) in TBS overnight at 4°C and blocked with TBS-CaM. After washing with TBS-Ca, 100 μL of 125I-labeled APC (1.87 μg/mL), protein C (3.60 μg/mL), protein S (0.17 μg/mL), C4BP (22.1 μg/mL), or MBL (21.2 μg/mL) in TBS-Ca were incubated for 4 hours at 37°C. Proteins were removed and wells were washed with ice-cold TBS-Ca four times. The amount of (A) 125I-APC, (B) 125I-protein C, (C) 125I-protein S, (D) 125I-C4BP, or (E) 125I-MBL binding was quantified by gamma-radiation counting and compared to the expected amount of binding of the unlabeled counterpart calculated using the equilibrium dissociation constant and the molecularity of the interaction. No statistical significant differences were seen.