ROLES OF PHOSPHATIDYLINOSITOL 4,5-BISPHOSPHATE IN FcεRI-MEDIATED SIGNALING IN MAST CELLS

A Dissertation
Presented to the Faculty of the Graduate School
of Cornell University
in Partial Fulfillment of the Requirements for the Degree of
Doctor of Philosophy

by
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Allergic reactions to substances such as pollen are initiated by immune cells called mast cells. Receptors expressed on the surface of mast cells, termed FcεRI, bind immunoglobulin E to detect these substances and thereby stimulate the calcium-dependent secretion of inflammatory mediators such as histamine (degranulation). In this dissertation we investigate two important events in mast cell signaling; FcεRI trafficking and phosphorylation, and the elevation in intracellular Ca$^{2+}$ levels, both of which are crucial for stimulated degranulation. In particular, we study the roles of the membrane glycerophospholipid, phosphatidylinositol 4,5-bisphosphate (PI(4,5)P$_2$), and two PI(4,5)P$_2$ synthesizing enzymes, namely the alpha and gamma isoforms of the type I phosphatidylinositol 4-phosphate 5-kinase family (PIP5kinase-I$_\alpha$ and -I$_\gamma$), in the above processes.

In response to FcεRI activation in mast cells, phospholipase C$\gamma$-mediated hydrolysis of PI(4,5)P$_2$ generates inositol 1,4,5-trisphosphate (IP$_3$), that triggers Ca$^{2+}$ release from the endoplasmic reticulum (ER) via IP$_3$ receptors. Depletion of ER stores activates influx of Ca$^{2+}$ by store-operated Ca$^{2+}$ entry mechanisms. In this study, we show that PIP5kinase-I$_\gamma$ contributes positively to the IP$_3$-mediated Ca$^{2+}$ release from the ER, whereas PIP5kinase-I$_\alpha$ negatively regulates store-operated Ca$^{2+}$ influx. Interestingly, both of these effects are mediated at the plasma membrane, suggesting spatially segregated synthesis of PI(4,5)P$_2$
by these PIP5kinase-I isoforms in regulating Ca\(^{2+}\) mobilization.

The surface expression and phosphorylation of Fc\(\epsilon\)RI is the first step in mast cell activation. Using chimeric receptors, we provide evidence for previously uncharacterized roles for negatively charged phospholipids in the ER and a polybasic sequence in the gamma subunit of Fc\(\epsilon\)RI in the regulation of trafficking of this receptor to the cell surface. We also show that PI(4,5)P\(_2\) regulates the stability of the receptor at the plasma membrane and its phosphorylation by Lyn kinase, potentially by binding to the polybasic sequence.
Lavanya was born to Kumudavalli and P. Vasudevan. Her childhood days were spent tagging along with her brother, climbing trees, and watching kung-fu movies. In school, she excelled in academics and extracurricular activities. Her love for biology and helping people exceeded her desire to pursue classical dance. So with the purpose of contributing to research for developing new therapies to human diseases, she graduated with a bachelor’s degree in pharmaceutical sciences. She then decided to come to Cornell University to expand her knowledge of cellular processes. Here, in the laboratory of Drs. Barbara Baird and David Holowka, she studied signaling pathways in allergies and the role of a functionally versatile class of lipids called phosphoinositides. At Cornell, Lavanya also met her husband, Ashwin. They plan to pursue research careers in sunny California in the coming years.
This dissertation is dedicated to my parents Dr. P. Vasudevan and Dr. Kumudavalli Vasudevan, my dear brother Aravindan, and my husband Ashwin, for believing in me all the way.
ACKNOWLEDGEMENTS

I am deeply indebted to Dave Holowka for being an incredible mentor. His patience and guidance has been invaluable. I have gained technical insights and sharpened my analytical skills thanks to his vast knowledge. I am thankful to Barbara Baird for always helping me focus on the big picture. She forced me to constantly think and re-evaluate my goals. Special thanks to my committee members Rick Cerione and Bill Brown for the valuable discussions and comments, as well as help with laboratory resources for experiments.

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Past members Ryan Young, Julie Gosse, Raibatak Das have been of great help and have patiently answered my questions about research even after their departure from the lab. Chikako Torigoe taught me many techniques and is a dear friend. I thank Stephanie, Ethan, Jinmin, Deepti, Kari, Amit, and Jon for their great spirit and for making the lab a fun place.

I am very grateful to all my friends who made my stay in Ithaca so enjoyable. My family has been my greatest motivation and my biggest supporter. My parents, have always led by example, being extremely devoted teachers and researchers throughout their careers. My brother has always believed that I can achieve more, and I owe a lot to him for pushing me to get this far.

Ithaca became a more beautiful place to live since I met Ashwin. He has been my greatest motivation through the tough times of thesis writing! Now as we march forward in our new journey to a new destination, I look forward to a future filled with exciting and happy times.
# TABLE OF CONTENTS

Biographical Sketch ........................................ iii
Dedication .................................................. iv
Acknowledgements .......................................... v
Table of Contents .......................................... vii
List of Figures ............................................ x
List of Abbreviations ....................................... xiii

1 Introduction

1.1 Mast cells participate in allergic and inflammatory conditions through the activation of IgE-receptors ............................. 2
1.2 PI(4,5)P₂ is a versatile signaling molecule .......................... 9
1.3 PI(4,5)P₂ levels in cells are tightly regulated by the type I PIP5kinases and inositol polyphosphate 5’-phosphatases .......................... 13
    1.3.1 Type I Phosphatidylinositol 4-phosphate 5-kinases .......................... 13
    1.3.2 Inositol Polyphosphate 5-phosphatases .......................... 22
1.4 Spatio-temporal regulation of PI(4,5)P₂ .......................... 23
1.5 Emerging role for PI(4,5)P₂ in the regulation of ion channels and surface receptors at the plasma membrane .......................... 26
1.6 Contributions .......................................... 28

Bibliography ............................................. 29

2 The alpha and gamma isoforms of PIP5kinase-I regulate distinct stages of calcium mobilization in mast cells

2.1 Abstract .................................................. 38
2.2 Introduction ............................................ 38
2.3 Materials and Methods ..................................... 42
    2.3.1 Materials ............................................ 42
    2.3.2 Cell culture and generation of stable cell lines ............................................ 42
    2.3.3 Immunocytochemistry ............................................ 43
    2.3.4 Immunoprecipitation ............................................ 44
    2.3.5 Measurement of PI(4,5)P₂ levels ............................................ 45
    2.3.6 Ca²⁺ measurements ............................................ 46
    2.3.7 Ruffling assay ............................................ 48
2.4 Results .................................................. 48
    2.4.1 Ectopically expressed PIP5kinase-Iα synthesizes a plasma membrane pool of PI(4,5)P₂ in RBL-2H3 cells .......................... 48
    2.4.2 PIP5kinase-Iα negatively regulates store-operated Ca²⁺ influx ............................................ 58
    2.4.3 Plasma membrane-associated yeast inositol polyphosphate 5’-phosphatase, Inp51, alters stimulated Ca²⁺ responses ............................................ 59
2.4.4 PIP5kinase-I\(\gamma\) contributes to antigen-mediated Ca\(^{2+}\) release from stores. .............................................. 64
2.4.5 Lipid raft-targeted depletion of PI(4,5)P\(_2\) enhances Ca\(^{2+}\) responses to antigen .................................................. 76
2.5 Discussion ..................................................................... 78
2.6 Summary and Proposed Mechanisms ................................. 86

Bibliography 88

3 Novel roles for a polybasic sequence in Fc\(\epsilon\)RI\(\gamma\)cyt in the regulation of surface expression and phosphorylation of the IgE-receptor 93
3.1 Abstract ........................................................................ 93
3.2 Introduction .................................................................... 94
3.3 Materials and methods ................................................... 99
  3.3.1 Materials ................................................................. 99
  3.3.2 Cell culture ............................................................. 99
  3.3.3 Transfection and Immunocytochemistry ....................... 99
  3.3.4 Cloning of \(\alpha\gamma\gamma\) ................................................. 100
  3.3.5 Mutagenesis of transmembrane sequence of \(\alpha\gamma\gamma\) ...... 102
  3.3.6 Cloning of ER-targeted constructs ............................. 104
  3.3.7 Tyrosine phosphorylation ......................................... 107
  3.3.8 Receptor endocytosis .............................................. 108
3.4 Results ........................................................................... 108
  3.4.1 PI(4,5)P\(_2\) positively regulates stimulated tyrosine phosphor-ylation of Fc\(\epsilon\)RI ......................................................... 108
  3.4.2 Depletion of PI(4,5)P\(_2\) at the plasma membrane accelerates actin-dependent internalization of Fc\(\epsilon\)RI. ............. 109
  3.4.3 Expression of \(\alpha\gamma\gamma\) constructs ............................ 115
  3.4.4 Expression of an ER targeted MARCKS effector domain blocks the export of wt \(\alpha\gamma\gamma\) receptors from the ER ....... 119
3.5 Discussion ...................................................................... 123
3.6 Proposed model ............................................................ 131

Bibliography 133

4 Summary and future directions 138
4.1 The big picture: an integrated model for the role of PI(4,5)P\(_2\) in mast cells .......................................................... 139
4.2 Future directions .......................................................... 141

Bibliography 147

A Additional results from PIP5kinase-I study 149
A.1 Distribution of endogenous PIP5kinase-I\(\alpha\) and \(\gamma\) by immunocytochemistry .................................................. 149
A.2 Summary of other conditions on the Ca$^{2+}$ response for PIP5kinase-I$\alpha$ constructs .................................................. 153
A.3 Summary of the effect of PIP5kinase-I$\alpha$ on degranulation ........ 160
A.4 Summary of degranulation and ruffling response for Inp cells ........ 161
A.5 Degranulation response for wt and PIP5kinase-I$\gamma^{-/-}$ BMMCs ... 164
A.6 Summary of total Ca$^{2+}$ response for cells overexpressing PIP5kinase-I$\gamma$ by flow cytometry .............................................. 169
A.7 Summary of ruffling response for cells overexpressing PIP5kinase-
I$\gamma$ ................................................................. 171

Bibliography .............................................................. 174

B Protocols ........................................................................ 176
B.1 Protocol for extracting bone marrow derived mast cells from newborn mice legs ...................................................... 176
B.1.1 Materials and Reagents .............................................. 176
B.1.2 Protocol ............................................................... 177
B.2 Small scale viral transfection using lentivirus ..................... 178
B.2.1 Safety requirements ................................................ 178
B.2.2 Cell culture- 293FT cells ........................................... 179
B.2.3 Lentivirus Infection Protocol - small scale ..................... 180
B.2.4 Cloning shRNAs into pLL3.7 ....................................... 183

Bibliography .............................................................. 187
## LIST OF FIGURES

1.1 Schematic illustration of events in an allergic reaction .......................... 3
1.2 FcεRI-mediated signaling pathways in mast cells. ................................. 6
1.3 Structure of phosphatidylinositol (PI) and phosphatidylinositol 4,5-bisphosphate (PI(4,5)P$_2$). ..................................................... 10
1.4 Enzymes in phosphoinositide metabolism. .......................................... 12
1.5 Schematic representation of murine PIP5kinase-Iα domain structure. ....... 17

2.1 Quantitative image analysis of DsRED-PLCδ PH domain recruitment to the plasma membrane. ......................................................... 47
2.2 Expression and subcellular localization of HA-tagged WT PIP5kinase-Iα and catalytically inactive mutant kinase by immunocytochemistry. ................................................................. 50
2.3 Expression of HA-tagged WT PIP5kinase-Iα and catalytically inactive mutant kinase by western blotting. ........................................... 51
2.4 Expression of transiently transfected DsRED-PLCδ-PH domain in 2H3 cells by confocal microscopy. ......................................................... 53
2.5 Representative plots for PH domain recruitment upon antigen stimulation. ........................................................................................................ 54
2.6 Representative plots for PH domain recruitment upon A23 stimulation. ........................................................................................................ 55
2.7 Expression of mutant PIP5kinase-Iα suppresses stimulated cell ruffling. ........................................................................................................ 56
2.8 Quantitation of stimulated ruffling for PIP5kinase-Iα overexpression. ........................................................................................................ 57
2.9 Ca$^{2+}$ response to antigen stimulation for 2H3, IαWT and IαMut cells. ........................................................................................................ 60
2.10 Ca$^{2+}$ response to antigen stimulation in the absence of extracellular Ca$^{2+}$ for 2H3, IαWT and IαMut cells. ........................................ 61
2.11 Ca$^{2+}$ response to stimulation by thapsigargin for 2H3, IαWT and IαMut cells. ........................................................................................................ 62
2.12 Quantitation of Ca$^{2+}$ response for 2H3, IαWT and IαMut cells. .......... 63
2.13 Expression and subcellular localization of ECFP-Inp51 compared to untransfected 2H3 cells by confocal microscopy. ......................... 65
2.14 Net decrease in PI(4,5)P$_2$ levels at the PM in cells overexpressing Inp51 observed upon stimulation with thapsigargin. ......................... 66
2.15 Enhanced Ca$^{2+}$ response to antigen for Inp cells compared to 2H3 cells. ........................................................................................................ 67
2.16 Enhanced Ca$^{2+}$ response to thapsigargin for Inp cells compared to 2H3 cells. ........................................................................................................ 68
2.17 Decreased antigen-stimulated Ca$^{2+}$ release from stores for Inp cells compared to 2H3 cells. ................................................................. 69
<table>
<thead>
<tr>
<th>Section</th>
<th>Title</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.18</td>
<td>Quantitation of Ca(^{2+}) response for Inp and 2H3 cells</td>
<td>70</td>
</tr>
<tr>
<td>2.19</td>
<td>Fc(\epsilon)RI expression on WT and PIP5kinase-I(\gamma^{-/-}) BMMCs.</td>
<td>72</td>
</tr>
<tr>
<td>2.20</td>
<td>Antigen-stimulated Ca(^{2+}) response for WT and PIP5kinase-I(\gamma^{-/-}) BMMCs.</td>
<td>73</td>
</tr>
<tr>
<td>2.21</td>
<td>Quantitation of the Ca(^{2+}) response for WT and PIP5kinase-I(\gamma^{-/-}) BMMCs.</td>
<td>74</td>
</tr>
<tr>
<td>2.22</td>
<td>Transient expression of WT and mutant PIP5kinase-I(\gamma^{87}) and -I(\gamma^{90}) in 2H3 cells.</td>
<td>75</td>
</tr>
<tr>
<td>2.23</td>
<td>Expression and subcellular localization of raft and non-raft targeted Inp54 in RBL-2H3 cells</td>
<td>77</td>
</tr>
<tr>
<td>2.24</td>
<td>Ca(^{2+}) response to antigen for cells expressing L10-GFP compared to untransfected cells.</td>
<td>79</td>
</tr>
<tr>
<td>2.25</td>
<td>Ca(^{2+}) response to antigen for cells expressing L10-Inp54-GFP compared to untransfected cells.</td>
<td>80</td>
</tr>
<tr>
<td>2.26</td>
<td>Quantitation of total Ca(^{2+}) response for cells expressing L10-GFP and L10-Inp54-GFP.</td>
<td>81</td>
</tr>
<tr>
<td>3.1</td>
<td>Schematic representation of the biosynthetic pathway for Fc(\epsilon)RI.</td>
<td>95</td>
</tr>
<tr>
<td>3.2</td>
<td>List of primers used for the cloning of (\alpha\gamma\gamma).</td>
<td>101</td>
</tr>
<tr>
<td>3.3</td>
<td>List of primers used for mutating polybasic residues in the juxtamembrane region of (\alpha\gamma\gamma).</td>
<td>103</td>
</tr>
<tr>
<td>3.4</td>
<td>ER-targeted MARCKS effector domain and control EGFP construct used in this study.</td>
<td>105</td>
</tr>
<tr>
<td>3.5</td>
<td>Primers used for cloning the ER-targeted MARCKS constructs.</td>
<td>106</td>
</tr>
<tr>
<td>3.6</td>
<td>Wt PIP5kinase-I(\alpha) expression enhances Fc(\epsilon)RI phosphorylation.</td>
<td>110</td>
</tr>
<tr>
<td>3.7</td>
<td>Mutant PIP5kinase-I(\alpha) expression inhibits Fc(\epsilon)RI phosphorylation.</td>
<td>111</td>
</tr>
<tr>
<td>3.8</td>
<td>Expression of ECFP-Inp51 reduces Fc(\epsilon)RI phosphorylation.</td>
<td>112</td>
</tr>
<tr>
<td>3.9</td>
<td>Stimulated endocytosis of IgE-Fc(\epsilon)RI is accelerated in Inp cells compared to 2H3 cells.</td>
<td>113</td>
</tr>
<tr>
<td>3.10</td>
<td>Inp cells show a 2.5 fold increase in receptor endocytosis levels compared to 2H3 cells.</td>
<td>114</td>
</tr>
<tr>
<td>3.11</td>
<td>Expression of wt (\alpha\gamma\gamma) in RBL-2H3 mast cells.</td>
<td>117</td>
</tr>
<tr>
<td>3.12</td>
<td>Expression of wt (\alpha\gamma\gamma) in CHO cells.</td>
<td>118</td>
</tr>
<tr>
<td>3.13</td>
<td>Wt and mutant (\alpha\gamma\gamma) constructs used in this study.</td>
<td>120</td>
</tr>
<tr>
<td>3.14</td>
<td>Expression of wt and mutant (\alpha\gamma\gamma) constructs in CHO cells in suspension.</td>
<td>121</td>
</tr>
<tr>
<td>3.15</td>
<td>Expression of wt and mutant (\alpha\gamma\gamma) constructs in attached CHO cells.</td>
<td>122</td>
</tr>
<tr>
<td>3.16</td>
<td>Coexpression of ER-localized MARCKS in CHO cells blocks the export of wt (\alpha\gamma\gamma) receptors.</td>
<td>124</td>
</tr>
<tr>
<td>3.17</td>
<td>Effect of the coexpression of ER-localized or plasma membrane localized MARCKS on the surface expression of wt (\alpha\gamma\gamma) receptors.</td>
<td>125</td>
</tr>
<tr>
<td>4.1</td>
<td>Summary of important results</td>
<td>140</td>
</tr>
</tbody>
</table>
A.1 Endogenous PIP5kinase-I\(\alpha\) is detected in the cytoplasm and the plasma membrane. ................................................. 151
A.2 Commercial antibody raised against PIP5kinase-I\(\alpha\) detects a pool that is predominantly plasma membrane localized. ....... 152
A.3 Endogenous PIP5kinase-I\(\gamma\) isoforms are at the plasma membrane, cytosol and nucleus in RBL-2H3 cells. ......................... 154
A.4 Effect of Gd\(^{3+}\) and U73122 on antigen-stimulated store-operated Ca\(^{2+}\) influx in RBL-2H3 cells. ............................... 156
A.5 Effect of Gd\(^{3+}\) on thapsigargin-mediated store-operated Ca\(^{2+}\) influx in RBL-2H3 cells. ................................. 157
A.6 Effect of high potassium concentration on antigen-stimulated Ca\(^{2+}\) response in RBL-2H3 cells. ................................. 158
A.7 Effect of potassium exclusion on thapsigargin-mediated Ca\(^{2+}\) response in RBL-2H3 cells. ............................. 159
A.8 Degranulation responses in I\(\alpha\)WT and I\(\alpha\)Mut clones, and 2H3 cells. .............................................................. 162
A.9 Degranulation response for Inp cells compared to 2H3 cells. .... 163
A.10 Stimulated ruffling in Inp cells. .................................................. 165
A.11 Stimulated ruffling is not inhibited in Inp cells. ...................... 166
A.12 Degranulation response for wt and PIP5kinase-I\(\gamma^{-/-}\) BMMCs. .. 168
A.13 Total Ca\(^{2+}\) response in cells expressing wt and mutant PIP5kinase-I\(\gamma^{87}\) and -I\(\gamma^{90}\) by flow cytometry. ..................... 170
A.14 Expression of wt or mutant PIP5kinase-I\(\gamma\) suppresses stimulated cell ruffling. .................................................. 172
A.15 Quantitation of stimulated ruffling in RBL-2H3 cells expressing wt or mutant PIP5kinase-I\(\gamma\). ............................... 173
# LIST OF ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>A23187</td>
<td>Calcium ionophore</td>
</tr>
<tr>
<td>Ag</td>
<td>Antigen or DNP-BSA</td>
</tr>
<tr>
<td>αγγ</td>
<td>Chimeric receptors containing extracellular domain of FcεRI alpha subunit, and transmembrane and cytoplasmic segments of FcεRI gamma subunit</td>
</tr>
<tr>
<td>BMMC</td>
<td>Bone Marrow Derived Mast Cells</td>
</tr>
<tr>
<td>Ca²⁺</td>
<td>Calcium</td>
</tr>
<tr>
<td>CHO</td>
<td>Chinese Hamster Ovary Cells</td>
</tr>
<tr>
<td>CRAC</td>
<td>Calcium Release Activated Current</td>
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<tr>
<td>DAG</td>
<td>Diacylglycerol</td>
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<td>DMEM</td>
<td>Dulbecco’s modified eagle medium</td>
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<td>DNP-BSA</td>
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<td>DsRED</td>
<td>Discosoma sp. red fluorescent protein</td>
</tr>
<tr>
<td>EGFP</td>
<td>Enhanced Green Fluorescent Protein</td>
</tr>
<tr>
<td>EGFR</td>
<td>Epidermal Growth Factor Receptor</td>
</tr>
<tr>
<td>ENaC</td>
<td>Epithelial Sodium Channel</td>
</tr>
<tr>
<td>ER</td>
<td>Endoplasmic Reticulum</td>
</tr>
<tr>
<td>EYFP</td>
<td>Enhanced Yellow Fluorescent Protein</td>
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<tr>
<td>FcεRI</td>
<td>High affinity receptor for immunoglobulin E</td>
</tr>
<tr>
<td>FcεRIγcyt</td>
<td>Cytoplasmic segment of FcεRI gamma subunit</td>
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<td>FCS</td>
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<td>GdCl₃</td>
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<td>RBL-2H3 cells stably expressing catalytically inactive (D203A) PIP5kinase-Iα</td>
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<td>IP₃</td>
<td>Inositol 1,4,5-trisphosphate</td>
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<td>ITAM</td>
<td>Immunotyrosine based activation motif</td>
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<tr>
<td>KCl</td>
<td>Potassium Chloride</td>
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<tr>
<td>KDEL</td>
<td>ER-retaining Lysine-Aspartate-Glutamate-Leucine sequence</td>
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<tr>
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<tr>
<td>L10-GFP</td>
<td>Membrane Anchoring Domain of Lck fused to GFP</td>
</tr>
<tr>
<td>MEM</td>
<td>Minimum Essential Medium</td>
</tr>
<tr>
<td>PA</td>
<td>Phosphatidic Acid</td>
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<tr>
<td>PDI</td>
<td>Protein Disulfide Isomerase</td>
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<tr>
<td>PH</td>
<td>Plekstrin Homology Domain</td>
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<tr>
<td>PI</td>
<td>Phosphatidylinositol</td>
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<td>Phosphatidylinositol 3,4,5-trisphosphate</td>
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<td>Phosphatidylinositol 4-phosphate</td>
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<td>PI(4,5)P2</td>
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<td>Phospholipase Cγ</td>
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<td>Plekstrin Homology domain from Phospholipase Cδ</td>
</tr>
<tr>
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</tr>
<tr>
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</tr>
<tr>
<td>S15-GFP</td>
<td>Membrane anchoring domain of Src fused to GFP</td>
</tr>
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<td>shRNA</td>
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<td>Short interfering Ribonucleic Acids</td>
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</tr>
<tr>
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</tr>
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<td>Stromal Interaction Molecule 1</td>
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<td>Canonical Transient Receptor Potential Channel</td>
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<td>TRPM</td>
<td>Transient Receptor Potential Melastatin</td>
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<td>U73122</td>
<td>Phospholipase C inhibitor</td>
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CHAPTER 1
INTRODUCTION

The National Institute of Allergy and Infectious Diseases defines allergy as a specific reaction of the body’s immune system to a normally harmless substance. Some commonly encountered allergic triggers (allergens) include pollen, pet dander, dust mites, insect venom, food substances, and drugs. The type I hypersensitivity reaction is the most common manifestation of allergic reactions, and is mediated by a special class of immunoglobulins termed IgE. The circulating level of IgE in the blood is normally very low (∼0.1µg/L) [57, 77]. However, in individuals exposed to allergens, IgE levels increase almost 10-fold and bind to receptors on specialized cells of the immune system, called mast cells (Figure 1.1). These cells are part of the first line of defense in the body, and are strategically located in mucous membranes lining the airway and the gut, as well as in other tissues. Subsequent exposure of previously sensitized mast cells to allergens activates divergent signaling cascades, and causes the release of preformed chemical mediators such as histamine. Depending on the nature of the mediators released and the severity of the response, the outcome may be limited to annoying symptoms such as sneezing, runny nose, itching and watery eyes, or escalate to potentially fatal consequences such as tissue damage and anaphylactic shock.

Historically, such IgE-associated responses are important in alerting the immune system to infections by bacteria, and parasites such as helminths. However, there is consensus in the medical field that most allergic reactions are inappropriate and often unnecessary responses of the immune system. Currently, one in three individuals is affected by allergic disorders. In fact, it is estimated
that on an average, about 40-50 million Americans suffer from allergies and associated diseases like asthma every year\textsuperscript{1}. Allergic reactions are especially dangerous in young children who may be incapable of recognizing symptoms and seeking care. Although we have a much improved understanding of factors that trigger and regulate mast cell activity, the prevention of allergic disorders still remains a challenge. Currently available over-the-counter anti-allergy medicines primarily treat the symptoms of allergy by blocking mediator release or activity. These inhibitory effects are not limited to affected tissues and are accompanied by side effects such as drowsiness, that hamper the quality of life of individuals. The humanized monoclonal antibody, Omalizumab (Novartis Pharmaceuticals Ltd.), that targets IgE \textsuperscript{27}, is a new drug currently being tested in clinical trials, and offers hope for preventing allergies by blocking the binding of IgE to mast cell receptors. Meanwhile, the search for stronger, more effective drugs continues. This will require identification of new cell-specific drug targets which may be used to prevent allergies rather than to merely treat the symptoms. Knowledge of the finer aspects of mast cell development and activation are crucial to achieve this goal.

1.1 Mast cells participate in allergic and inflammatory conditions through the activation of IgE-receptors

Mast cells are tissue-based effectors of allergic responses, characterized by the presence of dense cytoplasmic granules loaded with allergic mediators \textsuperscript{16, 57}. Mast cells arise from CD34\textsuperscript{+} stem cell progenitors in the bone marrow, and subsequently migrate to connective and mucosal tissues where they differentiate

\textsuperscript{1}“Airborne allergens: something in the air”, National Institute of Allergy and Infectious Diseases, 2003, NIH Publication No. 03-7045.
Figure 1.1: Schematic illustration of events in an allergic reaction. In response to pollen, plasma cells secrete IgE that binds to FcεRI on mast cells. Subsequent exposure to pollen causes crosslinking of FcεRI-IgE complexes and triggers degranulation, releasing chemical mediators that produce a variety of pharmacological effects as indicated.
under the influence of stem cell factor (SCF). Bone marrow derived mast cells as well as cultured mast cell lines such as Rat Basophilic Leukemia (RBL-2H3) cells are used as models to study the signaling pathways in allergies.

**FcεRI, the high affinity receptor for IgE, is expressed on the surface of mast cells**

The release of allergic mediators from mast cells requires the activation of surface receptors that bind IgE, termed FcεRI. Typically, mast cells express upwards of $10^5$ FcεRI receptors per cell [41], with the affinity of FcεRI for IgE ($\sim 10^{-10} \text{M}^{-1}$) being about 1000 times greater than that for the low affinity receptor for IgE, FcεRII (found on B-cells) [19, 57]. FcεRI belongs to the family of multichain immune recognition receptors (MIRRs), and T-cell and B-cell receptors for antigen are other prominent members of this family. MIRRs are characterized by the presence of separate ligand binding and signal transducing subunits, as well as conserved tyrosine motifs (termed immunotyrosine activation motifs or ITAMs). In rodents, FcεRI is expressed as a tetramer composed of an extracellular $\alpha$ subunit that binds IgE [20], a four transmembrane $\beta$ subunit that functions as a signal amplifier [44], and a disulfide linked dimer of $\gamma$ subunits that activates cellular signaling cascades. The $\beta$ and $\gamma$ subunits possess one ITAM each that act as sites for phosphorylation by Lyn kinase. FcεRI subunits undergo folding and assembly in the ER, following which the receptor is expressed at the plasma membrane. The human FcεRI$\alpha$ subunit is structurally related to rodent FcεRI$\alpha$ but does not require coexpression of FcεRI$\beta$ for surface expression [22]. Hence, in addition to the tetrameric version, human FcεRI is also expressed as an $\alpha\gamma_2$ trimer. This trimeric version is primarily found on
monocytes, Langerhans cells, and dendritic cells where it functions in antigen presentation via IgE [41]. Cell surface expression and signal transduction of \( \alpha \gamma_2 \) receptors are reduced compared to \( \alpha \beta \gamma_2 \) receptors [22, 44]. Mechanisms of assembly and transport of Fc\( \varepsilon \)RI are described in greater detail in Chapter 3.

**Phosphorylation of Fc\( \varepsilon \)RI by Lyn kinase is the first biochemically detectable step in mast cell activation.**

The activation of Fc\( \varepsilon \)RI occurs in two crucial steps: crosslinking-mediated association of clustered receptors with ordered microdomains in the plasma membrane, and phosphorylation of the \( \beta \) and \( \gamma \) subunit ITAMs by Lyn kinase. It is estimated that about 50% of the lipids in the plasma membrane are present in an ordered environment [28]. These ordered microdomains, also commonly referred to as lipid rafts, are enriched in glycosphingolipids and cholesterol. Experimental criteria used to define rafts include their insolubility in non-ionic detergents such as Triton X-100, and flotation as low density fractions on sucrose density gradients. Lyn-inactivating transmembrane phosphatases are excluded from rafts [79]. As a consequence, Lyn in lipid rafts has a 5-fold higher specific activity than Lyn in non-raft regions [78]. Antigen-mediated crosslinking of IgE-Fc\( \varepsilon \)RI complexes stabilizes their association with lipid rafts, allowing active Lyn in this environment to phosphorylate and bind to the Fc\( \varepsilon \)RI\( \beta \) ITAMs. Lyn then sequentially phosphorylates the ITAM sequences in the Fc\( \varepsilon \)RI\( \gamma \) subunit, and the \( \beta \) and \( \gamma \) subunits of adjacent receptors in the cluster [44, 66]. Gosse et al. demonstrated that transmembrane sequences of the receptor determined the capacity of the crosslinked receptors to associate with rafts and subsequently, the strength of downstream responses like degranulation [18]. The mechanism of
Figure 1.2: FcεRI-mediated signaling pathways in mast cells. A simplified view of the Lyn-dependent pathway is presented. Important steps leading to hydrolysis of PIP(4,5)P₂, and Ca²⁺ mobilization, are shown along with the three primary consequences of mast cell activation, namely degranulation, synthesis of cytokines, and membrane ruffling. Yellow stars represent phosphorylation.
crosslinking-driven receptor partitioning into rafts is not yet fully understood.

**Signaling events triggered by FcεRI activation**

Figure 1.2 depicts the main events in FcεRI signaling. Following phosphorylation of FcεRI subunits by Lyn, a cytosolic kinase, Syk, is recruited to FcεRIγ. Activated Syk phosphorylates several downstream targets including the Linker for the Activation of T-cells (LAT), and phospholipase Cγ (PLCγ). The hydrolysis of the membrane lipid phosphatidylinositol 4,5-bisphosphate (PI(4,5)P₂) by PLCγ generates soluble inositol 1,4,5-trisphosphate (IP₃) and membrane-bound diacylglycerol (DAG). IP₃ binds to its receptors in the ER and triggers the release of calcium from the ER stores whereas DAG activates protein kinase C (PKC). The ER-localized stromal interaction molecule 1 (STIM1) functions as a calcium sensor. Depletion of calcium from ER stores induces a conformational change in STIM1 that allows it to translocate to regions close to the plasma membrane. There it causes the oligomerization of the subunits of Orai1, a calcium release-activated channel. Calcium influx ensues to raise the intracellular calcium concentration. Activated PKC and calcium trigger the exocytotic release of granular contents to mediate the allergic response. In addition to Orai1, several members of the Transient Receptor Potential channel family (TRPC1, 3, 5, 7, and TRPM4) have been identified as modulators of calcium influx in mast cells [48, 72]. Thus far, roles for TRPC5 and TRPC1 have been suggested in the positive regulation of calcium influx in association with Orai1 [48], whereas TRPM4 channels have been shown to negatively regulate calcium influx in mast cells [72].
**Additional pathways:**

**Fyn:** A Lyn-independent pathway is proposed to be activated by another member of the Src-family of kinases, Fyn [52, 60]. Fyn phosphorylates the Grb-associated binder 2, Gab-2, that in turn stimulates phosphatidylinositol 3-kinase (PI3kinase) activity. PI3kinase-dependent kinase (PDK) activates Akt and the calcium independent PKCδ to stimulate degranulation.

**Rho family GTPases:** Rho family GTPases are low molecular weight GTP binding proteins that are very well characterized in their role in the assembly and organization of the actin cytoskeleton [21]. Activation of Rac or Cdc42 is important in FcεRI-mediated degranulation and calcium release [14, 31, 32]. In fact, a defect in the calcium and degranulation responses in a mutagenized variant of RBL cells, termed B6A4C1 (C1) cells, is rescued by the overexpression of dominant active forms of Rac and Cdc42 [14, 32].

**Actin cytoskeleton:** The actin cytoskeleton is a negative regulator of signaling events in mast cells. Inhibition of actin polymerization by cytochalasin D causes a more sustained antigen-mediated tyrosine phosphorylation of FcεRI subunits [29]. Moreover, FcεRI-mediated degranulation and calcium release are upregulated in the presence of cytochalasin D [15].

**PI(3,4,5)P₃:** PI(3,4,5)P₃ is generated by the phosphorylation of PI(4,5)P₂ by PI3kinase. Following receptor activation, PI3kinase is recruited to the plasma membrane, in part by binding to Btk [61]. PI(3,4,5)P₃ and Btk help recruit PLCγ to the plasma membrane where it hydrolyzes PI(4,5)P₂ to IP₃ and DAG. Stimulated synthesis of PI(3,4,5)P₃ is necessary for degranulation and calcium mobilization, as these responses are impaired when an inhibitor of PI3kinase, wort-
mannin, is applied to RBL-2H3 cells [67]. PI(3,4,5)P$_3$ also helps recruit Btk to the plasma membrane. SHIP, a phosphatase that dephosphorylates PI(3,4,5)P$_3$ to produce PI(3,4)P$_2$, dampens calcium and degranulation responses by preventing PI(3,4,5)P$_3$-mediated recruitment and activation of Btk and PLC$\gamma$ [59].

The roles of lipid intermediates in mast cell signaling are poorly understood compared to their protein counterparts. In subsequent sections, we describe a class of membrane lipids termed phosphoinositides, that have recently taken center stage in cellular signaling. We focus on PI(4,5)P$_2$, the classical substrate for PLC, and discuss its regulation and emerging roles in mast cell signaling.

1.2 PI(4,5)P$_2$ is a versatile signaling molecule

Phosphatidylinositol (PI), a constituent of cellular membranes, comprises of a water soluble inositol head group linked to two fatty acid chains by means of a glycerol moiety (Figure 1.3). Hokin and Hokin pioneered the study of this lipid species in the late 1950s by the discovery of phosphorylated derivatives of PI in a variety of tissues [23]. Since then, PI and its phosphorylated derivatives, collectively termed phosphoinositides, have been shown to possess distinct biological functions. The inositol head group of phosphatidylinositol can be reversibly phosphorylated on hydroxyl groups at the D3, D4, and D5 positions by a plethora of lipid kinases and phosphatases (Figure 1.4). Phosphoinositide derivatives are enriched in distinct cellular compartments for which they function as identification tags. For instance, PI(4,5)P$_2$ is enriched in the inner leaflet of the plasma membrane, PI and phosphatidylinositol 4-phosphate (PI4P) are localized to the Golgi, and phosphatidylinositol 3-phosphate (PI3P) acts as a
Figure 1.3: Structure of phosphatidylinositol (PI) and phosphatidylinositol 4,5-bisphosphate (PI(4,5)P$_2$). Phosphoinositides are present on cell membranes and comprise of a water soluble inositol head group linked to two fatty acid chains by means of a glycerol moiety. PI(4,5)P$_2$ is sequentially phosphorylated at the D4 and D5 positions by PI4kinase and PIP5kinase-I, respectively.
marker for endosomal compartments [9].

PI(4,5)P$_2$ is generated by the sequential phosphorylation of PI at the D4 and D5 positions by PI4kinases and PIP5kinases, respectively. It accounts for approximately 0.5-1% of the total membrane phospholipids [51]. PI(4,5)P$_2$ has garnered a lot of attention over the last decade due to its ability to regulate a variety of cellular events. Thus far, roles for PI(4,5)P$_2$ have been discovered in vesicle trafficking, phagocytosis [8], membrane ruffling [30], cell motility, cell adhesion [46], microtubule stabilization, and more recently in the regulation of ion channel activity and receptor phosphorylation [26]. In addition, PI(4,5)P$_2$ is the substrate for the generation of IP$_3$, DAG, and PI(3,4,5)P$_3$ [62]. It also functions as a signaling platform to recruit proteins to the plasma membrane [51].

PI(4,5)P$_2$ has a net negative charge of -4 that permits it to electrostatically interact with and recruit signaling intermediates that contain polybasic residues to the plasma membrane [50]. Recently, Heo et al. [24] demonstrated the recruitment of at least 37 different small GTPases by an interaction of their polybasic amino acid sequences with PI(4,5)P$_2$ and PI(3,4,5)P$_3$ at the plasma membrane. In PC12 cells, PI(4,5)P$_2$ is required for the regulated exocytosis of dense core vesicles [1]. In endocytosis, PI(4,5)P$_2$ binds and recruits clathrin adaptor proteins such as AP-2. Turnover of PI(4,5)P$_2$ also regulates membrane curvature, assisting in vesicle budding. PI(4,5)P$_2$ mediates actin polymerization by associating with multiple actin binding proteins [36]. For instance, it nucleates polymerization by recruiting the Wiscott-Aldrich syndrome protein (WASP) and thereby activating Arp2/3 complexes. It binds to and inactivates uncapping proteins such as profilin and gelsolin. PI(4,5)P$_2$ also regulates integrin-mediated signaling by activating the focal adhesion kinase.
Figure 1.4: **Enzymes in phosphoinositide metabolism.** Important mammalian (red) and yeast (blue) homologues of PI(P)kinases and 5-phosphatases are shown. Original nomenclature for the various kinase families is shown in *italics.*
Despite the many operations that consume PI(4,5)P$_2$, its overall concentration in the cells remains fairly constant due to compensatory synthesis by PIPkinases. A key step in understanding the functional diversity of PI(4,5)P$_2$ requires the knowledge of how PI(4,5)P$_2$ levels are regulated in the cell.

1.3 PI(4,5)P$_2$ levels in cells are tightly regulated by the type I PIP5kinases and inositol polyphosphate 5'-phosphatases.

1.3.1 Type I Phosphatidylinositol 4-phosphate 5-kinases.

In the search to isolate enzymes that synthesize PI(4,5)P$_2$, Bazenet et al. [4] purified two distinct PIPkinases from the erythrocyte membrane that they named as type I and type II PIPkinase, respectively. Originally, both enzymes were thought to use PI4P as a substrate. Rameh et al. [58] later demonstrated that the type II PIPkinase actually utilized PI5P as a substrate and was hence, a phosphatidylinositol 5-phosphate 4-kinase. Subsequently, the type I and II PIPkinases were renamed as PIP5kinase (referred to as PIP5kinase-I in this dissertation) and PIP4kinase to reflect their substrate preferences (Figure 1.4).

**PIP5kinase-I isoforms**

The type I family of PIP5kinases (PIP5kinase-I) are ATP-dependent enzymes that catalyze the final step in PI(4,5)P$_2$ synthesis by phosphorylating the D5 position on the myo-inositol ring of phosphatidylinositol 4-phosphate in vivo (Figure 1.4). These enzymes display greater promiscuity in vitro, phosphorylat-
ing the D3, D4 and D5 positions of the inositol ring [42]. Since the establishment of the type I enzyme as the dominant PIP5kinase, three isoforms, namely alpha, beta, and gamma as well as three alternatively spliced variants for the gamma isoform (-Iγ87, -Iγ90 and -Iγ93) have been described. Ishihara et al. cloned the murine versions of the three isoforms of PIP5kinase-I from a MIN6 insulin secreting cell line in 1997 [33, 34]. PIP5kinase-Iα encodes a 539 amino acid sequence of which approx 380 amino acids in the central region form the kinase core (see Figure 1.5) [34]. PIP5kinase-Iβ encodes a 546 amino acid sequence, with significant homology in the central kinase core domain with PIP5kinase-Iα, but with divergent amino and carboxy-terminus sequences. Both of these isoforms have a predicted molecular weight of approx 68KDa. PIP5kinase-Iγ87 encodes 635 amino acids, while PIP5kinase-Iγ90 encodes an additional 26 amino acid insert at the C-terminus. PIP5kinase-Iγ93 has a 26 amino acid insert N-terminal to that for γ90. PIP5kinase-Iγ is enriched in the brain, suggesting a role for this kinase isoform in neuronal regulation [10, 40]. PIPkinase activities are evolutionarily well-conserved [69]. The yeast homologue of PIP5kinase [47], MSS4 (Multicopy suppressor of stt4-1ts), is the primary source of PI(4,5)P2, and is localized to the plasma membrane in yeast [69]. Due to the high homology between their kinase core domains, growth defects in mss4 null yeast can be rescued by the overexpression of mammalian isoforms of PIP5kinases but not the PIP4kinases [43]. This property has been used to distinguish the activity of PIP4kinases from that of PIP5kinases. A role for Mss4p has been proposed in the regulation of actin polymerization in yeast [23].
Nomenclature

The traditional designations for the mouse and human homologues of PIP5kinase-I are switched: murine PIP5kinase-I\(\alpha\) is equivalent to human PIP5kinase-I\(\beta\), and vice versa. In addition, the murine splice variants are sometimes described based on their respective molecular weights as \(\gamma_{87}\), \(\gamma_{90}\) and \(\gamma_{93}\), and sometimes based on their amino acid lengths as \(\gamma_{635}\), \(\gamma_{661}\) and \(\gamma_{687}\), where the human homologue of \(\gamma_{635}\) is \(\gamma_{640}\), and that for \(\gamma_{661}\) is \(\gamma_{668}\). Recently, in an effort to bring uniformity, the entries for rodent PIP5kinase-\(\alpha\) and -I\(\beta\) in the NCBI database\(^2\) have been switched to conform to the human designations. Henceforth, in this dissertation, we will adopt the traditional murine nomenclature for PIP5kinase-I isoforms. Splice variants of PIP5kinase-I\(\gamma\) will be referred to as PIP5kinase-I\(\gamma_{87}\), \(\gamma_{90}\) and \(\gamma_{93}\) [40].

Structure of PIPkinases

The PIP5kinases form a unique class of lipid kinases. They share significant sequence and structural homology (~80%) with family members but show significant diversity from other PIPkinase families (only ~30% with PIP4kinase) and protein kinases [35]. The only PIPkinase structure known so far is that for PIP4kinase-\(\beta\), a flat disc shaped homodimer that has a large membrane interface interspersed with basic residues. The kinase domains of all PIPkinases, including yeast homologues like Mss4p, show conserved residues that are part of the active site. In this manner, although PIPkinases do not share any significant homology with protein kinases, their kinase core domains show similarity in structural folds [6, 47].

Conserved residues of PIP5kinase-Iα proposed to be important for its kinase activity are shown in Figure 1.5. The functions of these residues are proposed to be analogous to their counterparts in protein kinases. The G-loop region (GASGS) holds ATP in place during catalysis. Lys-138 bridges to the α and β phosphates of ATP, while the Mg\(^{2+}\) bound to Asp-350 chelates the γ phosphate. Asp-266 functions as a weak base, removing the proton from the target hydroxyl group of the inositol headgroup. This allows the bond between the γ phosphate and hydroxyl moiety to form. In addition, a conserved Asp-203 is thought to function in substrate specificity. Mutations in residues of PIP4kinase-β corresponding to Lys-138, Asp-266 or Asp-350 of PIP5kinase-I reduce the catalytic activity of the kinase. Ishihara et al. [34] reported that alanine mutations of the Gly-124 in the G-loop reduced the kinase activity of PIP5kinase-Iα by 40% compared to the wt kinase. In addition, mutation of the ATP-binding Lys-138 completely abolished activity.

Type I and type II PIPkinases have a 25 amino acid activation loop that is analogous to the loop found in protein kinases. Kunz et al. [42, 43] used loop swap mutants to show that this activation loop regulates substrate specificity and binding of PIPkinases. In fact, the substrate preference of PIP5kinase-I could be altered to that of a type II kinase by mutating a single conserved glutamate residue (E362) in the C-terminus of the activation loop. One interesting consequence of this mutation was the reduced association of the type I kinase with the plasma membrane, suggesting that the activation loop was also partly responsible for the membrane targeting of PIP5kinase-I.
Figure 1.5: Schematic representation of murine PIP5kinase-Iα domain structure. Residues implicated in PIP5kinase-Iα function and activation are highlighted. ND is the N-terminal domain, KD is the kinase core domain, AL is the activation loop (substrate specificity loop), and CD is the C-terminal domain.
Modulators of PIP5kinase-I activity

Protein kinases, phosphatidic acid, phospholipase D, Arf GTPases and Rho-family GTPases are regulators of PIP5kinase-I activity.

**Phosphorylation:** Itoh et al. [35] showed that PIP5kinase-I has protein kinase activity and is capable of autophosphorylation on serine residues. They later demonstrated using mutational analysis that phosphorylation of Ser-214 of PIP5kinase-I reduced its catalytic activity, suggesting that this is the site for autophosphorylation. The current model for PIP5kinase-I activation suggests that PIP5kinase-I is maintained in an inactive state by its autophosphorylation or phosphorylation by protein kinase A, at Ser214. The kinase becomes activated when it is dephosphorylated by a PKC dependent pathway, potentially due to activation of phosphatases such as PP1. On similar lines, Ling et al. [46] showed that the focal adhesion kinase (FAK) caused a 4-fold increase in phosphorylation of PIP5kinase-Iγ90 and its association with talin. Phosphorylation of PIP5kinase-Iγ90 at Tyr-644 increases interaction with talin while that at Ser-645 decreases interaction.

**Phosphatidic acid:** Phosphatidic acid (PA) is produced by the hydrolysis of phosphatidylcholine by Phospholipase D (PLD) or by the phosphorylation of diacylglycerol. The type I PIPkinases were initially classified based on their sensitivity to PA, whereas the type II kinase showed no sensitivity towards PA [38]. The activity of PIP5kinase-Iα and -Iβ were found to be enhanced up to 10-fold in the presence of PA [33]. PIP5kinase-Iγ was found to be the most sensitive, showing up to a 13-fold increase in activity to PA stimulation [34]. More recently, the C-terminal domain regions of PIP5kinase-Iβ containing the activation loop and the catalytic loop responsible for binding PI4P have been demonstrated to bind
PA [37]. This association involves both electrostatic interactions with the ionic phosphate in the head group as well as hydrophobic interactions with the unsaturated acyl chain of PA. Agonist-stimulated production of PA enhances the binding of PI4P to the active site of PIP5kinase-\(\beta\) and hence, overcomes the uncompetitive inhibition by PI4P resulting in PI(4,5)P\(_2\) synthesis.

**Phospholipase D:** Phospholipase D is activated in response to PKC. Both isoforms of phospholipase D, PLD1 and PLD2, can associate with PIP5kinase-I\(\alpha\), but only the activity of the plasma membrane-associated PLD2 is enhanced by PI(4,5)P\(_2\) in vivo [12]. PLD and PIP5kinase-I\(\alpha\), therefore, participate in a positive feedback loop wherein PI(4,5)P\(_2\) generated by PIP5kinase-I\(\alpha\) activates PLD2 to produce PA which in turn enhances PIP5kinase-I activity.

**Arf GTPases:** The ADP Ribosylation Factor (Arf) family of small GTPases has 6 mammalian isoforms: Arf 1-6 [13]. Arf6 directly interacts with PIP5kinase-I\(\gamma\) in a calcium-dependent manner [1]. In addition, Arf6 colocalizes with PIP5kinase-I\(\alpha\) and PLD1 in EGF-stimulated membrane ruffles in HeLa cells [30]. It is hence thought to control the localization and activation of the kinase isoforms at the plasma membrane, in the presence of PA. Arf1 has also been proposed to directly interact with and recruit PIP5kinase-I to the golgi [30, 39]. The identity of the isoform(s) involved in the synthesis of the PI(4,5)P\(_2\) pool in the golgi and the role for PI(4,5)P\(_2\) in this organelle are yet to be elucidated.

**Rho family GTPases:** Rho-family GTPases stimulate PIP5kinase-I activity whereas the Ras GTPases (RalA and Rap1A) have no effect on PI(4,5)P\(_2\) production [70, 71, 75]. RhoA and Rac associate with all three isoforms of PIP5kinase-I in vivo and in vitro in a GTP-independent manner suggesting an indirect mechanism of regulation [40]. Cdc42 potentiates the PIP5kinase activity of all three
isoforms but fails to show direct association with the enzymes. The C-terminus of Rac associates with PIP5kinase-\(I_\alpha\) and this interaction is necessary for actin polymerization. Ectopically expressed wt PIP5kinase-\(I_\alpha\) can rescue actin polymerization in cells expressing dominant negative Rho (RhoN19) [64], suggesting that Rho acts upstream from PIP5kinase-\(I_\alpha\).

**Plasma membrane targeting of PIP5kinase-I**

The plasma membrane targeting of PIP5kinase-I is regulated by the activation loop, a dibasic sequence in the kinase domain, and C-terminal motifs that allow interactions with unique partner proteins. The activation loop regulates substrate specificity and allows PIPkinase-I isoforms to bind to PI4P. In this manner, it directs PIP5kinase-I (PM) and PIP4kinases (cytoplasmic) to different cellular destinations. In agreement, Shibasaki et al. [64] suggested that the \(YR(X)_2SWK\) sequence in the activation loop was similar to the putative \(\text{PI}(4,5)\text{P}_2\) binding site of \(\alpha\)-actinin. Arioka et al. showed that mutation of the dilysine motif at the C-terminal end of the kinase domain reduced the plasma membrane association of PIP5kinase-\(I_\gamma\) [2]. Binding of a \(\text{WVYSPL}\) sequence in 28 amino acid extension of PIP5kinase-\(I_\gamma90\) to the FERM domain of talin causes the recruitment of the kinase to focal adhesions [11, 46]. The resultant localized production of \(\text{PI}(4,5)\text{P}_2\) causes the recruitment of other focal adhesion proteins such as vinculin and FAK in the generation of focal adhesion plaques. In contrast, PIP5kinase-\(I_\gamma87\) lacks the C-terminal extension and is more uniformly distributed at the plasma membrane [11].
Murine models for PIP5kinase-I

The phenotypes of PIP5kinase-I\(\alpha\) and -I\(\gamma\) knockout in mice are summarized below. PIP5kinase-I\(\beta^{-/-}\) mice have not been described yet.

**PIP5kinase-I\(\alpha^{-/-}\) mice:** Sasaki et al. [62] generated and characterized PIP5kinase-I\(\alpha^{-/-}\) mice in 2005. These mice develop normally and are healthy. Bone marrow derived mast cells (BMMCs) from PIP5kinase-I\(\alpha^{-/-}\) mice lack PIP5kinase-I\(\alpha\), but show normal expression of the -I\(\beta\) and -I\(\gamma\) isoforms, and Fc\(\epsilon\)RI. In addition, mast cell responses such as degranulation and calcium release are enhanced, and the KO mice suffer from more severe cutaneous and systemic anaphylactic responses compared to Wt mice. Surprisingly, despite a decrease in PI(4,5)P\(_2\) levels, PIP5kinase-I\(\alpha^{-/-}\) BMMCs show an increase in the levels of IP\(_3\) and PI(3,4,5)P\(_3\), suggesting that the pool of PI(4,5)P\(_2\) synthesized by PIP5kinase-I\(\alpha\) is dispensible as a substrate for the production of these two second messengers. Stimulated actin polymerization is reduced by almost 80% in KO mice. However, inhibition of actin polymerization in wt BMMCs by pretreatment with latrunculin only mimics the enhancing effects seen for degranulation in KO BMMCs and not on calcium mobilization in these cells. The association of Fc\(\epsilon\)RI with lipid rafts is also enhanced somewhat in the KO cells in an actin-independent manner. In summary, results from PIP5kinase-I\(\alpha^{-/-}\) mice suggests that this isoform serves as a negative regulator of mast cell activation, potentially by suppressing receptor activation.

**PIP5kinase-I\(\gamma^{-/-}\) mice:** Two groups have independently described PIP5kinase-I\(\gamma^{-/-}\) mice [10, 74]. The KO strain developed by Wang et al. [74] shows embryonic lethality by day 12. The cardiovascular development in these KO mice is severely impaired and proposed to be the cause of death. In contrast, the KO
line developed by Di Paolo et al. [10] dies within 24 hrs post birth, due to neurological and behavioral defects manifested as an inability to feed or move. The primary phenotype of these mice is impaired synaptic vesicle trafficking, and synaptic transmission in the brain. The defect in trafficking is also observed in chromaffin cells where the exocytosis of large dense core vesicles is impaired.

In summary, studies with these murine models for PIP5kinase-I strongly suggests non-redundancy in the biological roles of individual isoforms.

1.3.2 Inositol Polyphosphate 5-phosphatases

Inositol polyphosphate 5-phosphatases (IPP5P) are enzymes that dephosphorylate the D5 position of the inositol ring of soluble inositols IP$_3$ and inositol 1,3,4,5-tertrakis phosphate (IP$_4$), as well as membrane bound PI(4,5)P$_2$ and PI(3,4,5)P$_3$ [49]. Mammalian IPP5Ps are classified into four groups (I-IV) based on their substrate specificities and contain two signature motifs that are important for catalysis. Group I phosphatases use IP$_3$ and IP$_4$ as substrates. Group II enzymes dephosphorylate all D5 phosphorylated substrates including PI(4,5)P$_2$. Synaptojanin 1 and 2 are important members of this class and have been extensively studied for their role in synaptic vesicle recycling [3]. A prominent feature of synaptojanins is the presence of a yeast SacI enzyme like domain. This domain has been implicated in the broader substrate specificities of synaptojanin and its yeast orthologs (see following section). OCRL-1 (Oculocerebrorenal dystrophy-linked), found on lysosomes, is another group II enzyme. Group III enzymes, SHIP and SHIP2, dephosphorylate IP$_4$ and PI(3,4,5)P$_3$, and play a negative regulatory role in mast cell signaling [7, 3]. Group IV phosphatases
have not been characterized. Many diseases associated with PI metabolism are linked to the IPP5P enzymes: Lowe’s syndrome (OCRL), Diabetes (SHIP2) and cancer susceptibility (SHIP1) are examples [53].

Phosphatases in the budding yeast Saccharomyces cerevisiae are classified into three groups: Class I phosphatases contain sac-like domains, Class II phosphatases contain an inositol polyphosphate 5-phosphatase domain, while class III phosphatases are homologous to mammalian myotubularins [69]. Gene products of the INositol polyphosphate 5-Phosphatase family include INP51, 52, 52 and 54. All except Inp54p contain both sac- and the phosphatase domains and are orthologs of mammalian synaptojanin. The sac domain of Inp51 is inactive, and thus, this enzyme is specific for PI(4,5)P$_2$ [68]. Inp52p and Inp53p have more redundant roles due to their active sac domains, and dephosphorylate PI3P, PI4P, PI(3,5)P$_2$ and PI(4,5)P$_2$. Inp54 is also specific for PI(4,5)P$_2$. A role for Inp54 has been proposed in secretion [76]. Inp51p, 52p and 53p do not play a role in secretion but are important in actin polymerization, endocytosis, and cell wall maintenance. In contrast to Inp52p and Inp53p which show a more diffuse cytosolic localization, Inp54p is post translationally anchored to the ER via its C-terminal hydrophobic tail region. The subcellular localization of Inp51p is not well defined. Due to their specificity for PI(4,5)P$_2$, Inp51 and 54 are preferred over the mammalian group II enzymes in the study of PI(4,5)P$_2$ functions.

1.4 Spatio-temporal regulation of PI(4,5)P$_2$.

How does PI(4,5)P$_2$ regulate multiple cellular events? The answer potentially lies in the spatio-temporal regulation of PI(4,5)P$_2$ turnover. Based on the non-
redundancy in the functions of PIP5kinase-I isoforms, the idea that there may be multiple pools of PI(4,5)P_2 is gaining popularity.

**Local Synthesis of PI(4,5)P_2:** While it is difficult to visualize localized changes in PI(4,5)P_2 levels experimentally, there is growing evidence in the literature for such spatial synthesis of PI(4,5)P_2. The various PIP5kinase-I isoforms can interact with different effector proteins during cell activation. It is hypothesized that these interactions enable the translocation of the PIP5kinase isoforms to different compartments, or to different supersignaling complexes in the cell, to generate functionally specialized “pools” of PI(4,5)P_2. For instance, PIP5kinase-Iα has been shown to colocalize with Arf6 in membrane ruffles [30]. Yet another example is the binding of the 26 amino acid insert at the C-terminus of PIP5kinase-Iγ to talin in focal adhesions, and more recently, to the clathrin adaptor, AP-2 at the sites of endocytosis [46, 45]. In vivo, only about 0.3% of the total PIP5kinase activity is associated with Rac, suggesting that PIP5kinases may interact with other partner proteins and thus, offer a mechanism to localize PI(4,5)P_2 production [70]. Local synthesis of PI(4,5)P_2 may also cause membrane curvature and trap the phospholipid in structures like the phagocytic cup [8]. In fact, a transient association of PIP5kinase-Iα and increase in PI(4,5)P_2 levels can be detected in the phagosome boundary in macrophages.

**Association with PI(4,5)P_2-binding proteins (PIPmodulins):** In a recent study, Golebiewska et al. [17] showed using fluorescence correlation spectroscopy that the diffusion coefficient of fluorescent PI(4,5)P_2 in the inner leaflet of the plasma membrane in fibroblasts was 2-3 fold lower than that in the outer leaflet. Diffusion was also reduced compared to PI(4,5)P_2 in blebs and compared to other lipids like rhodamine-PE on the inner leaflet, suggesting that close to
2/3rds of the PI(4,5)P$_2$ in cells was reversibly associated with cellular proteins or ordered lipid domains. This is an important finding because it confirms that the diffusion of PI(4,5)P$_2$ away from the sites of synthesis may indeed be slower than expected, allowing local PI(4,5)P$_2$ gradients to form.

McLaughlin and Murray [50] suggest that any peptide containing a basic cluster of residues with a net charge of +4 or more is capable of laterally sequestering PI(4,5)P$_2$ (net charge = -4). The Myristoylated Alanine-Rich C-Kinase Substrate (MARCKS), a substrate for protein kinase C, is the prototype for such interactions. MARCKS possesses a central conserved cluster of basic residues (net charge = +13) that can electrostatically sequester up to 3 molecules of PI(4,5)P$_2$ and reduce its diffusion by 10-fold [50, 51]. It has been reported that in PC/PI(4,5)P$_2$ vesicles, the PI(4,5)P$_2$ bound to MARCKS is still available to bind the PLC$\delta$-PH domain suggesting a mechanism wherein locally concentrated PI(4,5)P$_2$ is available for interaction with effector proteins [51]. Other basic peptides capable of laterally sequestering PI(4,5)P$_2$ (also called PIPmodulins) include Growth Associated Protein (GAP43) and the NMDA receptor. Due to the electrostatic nature of their interactions, these PI(4,5)P$_2$-binding proteins release PI(4,5)P$_2$ in response to calcium mobilization and competition with Ca$^{2+}$-calmodulin. Phosphorylation of 3 serine residues in the effector domain of MARCKS also weakens binding to PI(4,5)P$_2$ [51].

**Synthesis in plasma membrane raft regions:** Lipid rafts have been proposed as preferential sites for the agonist-mediated turnover of phosphoinositides. Much of the evidence for the turnover of PI(4,5)P$_2$ in rafts comes from cholesterol depletion studies. For instance, caveolae are detergent resistant domains that are rich in glycosphingolipids and cholesterol, and can be isolated in low den-
density fractions in sucrose gradients. In A431 cells, hormone-stimulated PI(4,5)P₂ turnover depends on the integrity of caveolar structures [54]. Disruption of these domains by the chelation of cholesterol causes the loss of PI(4,5)P₂ from low density fractions, abrogates signaling, and inhibits the turnover of PI(4,5)P₂. These effects are reversed, and PI(4,5)P₂ re-compartmentalizes to caveolae when cholesterol is restored in these cells. Increased PI(4,5)P₂ levels and agonist-mediated synthesis of PI(3,4,5)P₃ that are reversibly sensitive to cholesterol depletion have also been detected in rafts isolated from platelets [5].

Other mechanisms such as the presence of an actin fence that restricts the lateral diffusion of membrane lipids, and direct hand-off of newly synthesized PI(4,5)P₂ to effector proteins have also been suggested [25]. The above models seem plausible and may occur either independently or in combination to regulate gradients of PI(4,5)P₂. However, more conclusive experiments, and newer methods to distinguish these pools are required to demonstrate the existence of multiple spatially segregated PI(4,5)P₂ pools in vivo.

1.5 Emerging role for PI(4,5)P₂ in the regulation of ion channels and surface receptors at the plasma membrane

Recent studies have uncovered new roles for PI(4,5)P₂ in the modulation of ion channels as well as cell surface receptors. Some well known examples of ion channels regulated by PI(4,5)P₂ include the inward rectifying calcium channels, the sodium-calcium exchanger, IP₃ and ryanodine receptors, voltage gated calcium channels, TRP channels, and epithelial sodium channel [26, 73]. In addition, PI(4,5)P₂ has also been shown to bind cytoplasmic domains of receptors
including TCRζ, FcεRIγ, and the EGF receptor [63, 65]. These channels and receptors are proposed to preferentially bind to PI(4,5)P_2 compared to other negatively charged phospholipids, due to its high charge density (-4 at pH=7). Preference over PI(3,4,5)P_3 is attributed to its higher relative abundance in cells [26].

In all reported instances, mechanisms involve the binding of a polybasic region on the channel or receptor to PI(4,5)P_2 [73]. For example, Sato et al. [63] reconstituted the transmembrane-juxtamembrane region of the EGF receptor in vesicles and monitored its interaction with PI(4,5)P_2 using FRET. The fluorescence signal increased upon addition of PI(4,5)P_2 to the vesicles and decreased in the presence of Ca^{2+}-calmodulin (+16), suggesting an electrostatic mechanism. In addition, circular dichroism spectra revealed that this juxtamembrane region was unstructured and flexible. Hence, the basic residues in the juxtamembrane region of the EGFR were proposed to associate with the negatively charged plasma membrane and sequester the kinase domains to prevent autophosphorylation. Subsequently, the agonist-mediated dimerization of the receptors facilitates the release of the kinase domains by binding to Ca^{2+}-calmodulin, and allows receptor activation. In the case of NMDA receptor, Ca^{2+}-calmodulin causes channel inactivation [50].

In the case of the epithelial sodium channel (ENaC), Pochynyuk et al. [55, 56] identified independent binding sites for PI(4,5)P_2 and PI(3,4,5)P_3 comprising of basic clusters of amino acids. PI(4,5)P_2 levels regulated the basal activity of the channel, whereas PI(3,4,5)P_3 caused an acute increase in channel activity in response to agonist stimulation. The PI(4,5)P_2-binding basic residues in this case were proposed to be inhibitory to channel opening, and binding to PI(4,5)P_2 was suggested to relieve the inhibition.
1.6 Contributions

Current studies suggest that the three PIP5kinase isoforms play non-redundant roles in PI(4,5)P$_2$ generation. In this dissertation, we describe a novel dual-regulatory mechanism for PI(4,5)P$_2$ in the mast cell calcium response (Chapter 2). First, using bone marrow derived mast cells from PIP5kinase-I$\gamma^−/−$ mice, we show that this isoform contributes to the PLC$\gamma$ hydrolyzable pool of PI(4,5)P$_2$ in mast cells. Second, we show that PI(4,5)P$_2$ synthesized by PIP5kinase-I$\alpha$ negatively regulates calcium influx. Furthermore, overexpression of the plasma membrane-localized PI(4,5)P$_2$-specific phosphatase from yeast, Inp51, reduces calcium release from the ER but enhances calcium influx, suggesting that both PIP5kinase-I$\alpha$ and -I$\gamma$ exert their effects at the plasma membrane. Based on the enhancement of calcium response by the depletion of PI(4,5)P$_2$ pools specifically associated with lipid rafts, we predict that PIP5kinase-I$\alpha$ preferentially localizes to these microdomains. This data provides evidence to support spatial segregation and distinct biological roles of PI(4,5)P$_2$ pools at the plasma membrane.

Chapter 3 describes a novel role for PI(4,5)P$_2$ in the regulation of IgE-receptor phosphorylation and trafficking. Ectopic expression of PIP5kinase-I$\alpha$ enhances receptor phosphorylation levels while the expression of Inp51 reduces phosphorylation. We suggest potential mechanisms by which a polybasic stretch of residues situated proximal to the plasma membrane in the cytoplasmic domain of Fc$\epsilon$RI$\gamma$ may be involved in regulating this effect. Using site-directed mutagenesis, we hypothesize a potential role for this sequence in the export of newly synthesized Fc$\epsilon$RI complexes from the ER.
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CHAPTER 2
THE ALPHA AND GAMMA ISOFORMS OF PIP5KINASE-I REGULATE DISTINCT STAGES OF CALCIUM MOBILIZATION IN MAST CELLS

2.1 Abstract

Antigen-mediated activation of mast cells triggers an increase in intracellular Ca\(^{2+}\) levels to support degranulation and release of chemical mediators in allergic and inflammatory conditions. Ca\(^{2+}\) is mobilized in two stages: release from stores in the endoplasmic reticulum, and influx from the external milieu. In this study, we show new roles for two phosphatidylinositol 4,5-bisphosphate (PI(4,5)P\(_2\)) synthesizing enzymes, namely the type I phosphatidylinositol 4-phosphate 5-kinase alpha and gamma (PIP5kinase-I\(\alpha\) and -I\(\gamma\)), in the regulation of Ca\(^{2+}\) mobilization in mast cells. We show that PIP5kinase-I\(\gamma\) contributes positively to Ca\(^{2+}\) release from ER stores, whereas PIP5kinase-I\(\alpha\) negatively regulates Ca\(^{2+}\) influx. In addition, we show that both of these PIP5kinase-I isoforms act at the plasma membrane in mast cells, and we provide evidence that PIP5kinase-I\(\alpha\) synthesizes PI(4,5)P\(_2\) that functions in ordered lipid microdomains in these cells.

2.2 Introduction

Ca\(^{2+}\) fluxes are observed in diverse signaling systems and regulate processes ranging from fertilization to smooth muscle contraction [32]. In this sense, Ca\(^{2+}\) functions as a universal second messenger. In mast cells, antigen-stimulated
increase in intracellular Ca\(^{2+}\) concentration is essential for the release of allergic mediators, and the secretion of cytokines. The endoplasmic reticulum (ER) is the primary organelle for Ca\(^{2+}\) storage in unstimulated cells. Recently, the mitochondria has become significant as a Ca\(^{2+}\) storage site under stimulating conditions [31]. The ER, however, is semi-permeable to Ca\(^{2+}\), allowing it to leak into the cytoplasm. The sarcoplasmic endoplasmic reticulum Ca\(^{2+}\) ATPase (SERCA), a Ca\(^{2+}\) uptake pump, refills ER stores and maintains the cytosolic Ca\(^{2+}\) concentrations at low levels in resting cells. The ER also hosts the channel for Ca\(^{2+}\) release, the inositol 1,4,5-trisphosphate receptor (IP\(_3\)R), that is selectively activated in response to antigen stimulation, raising the concentration of Ca\(^{2+}\) from 100nm in resting cells to ≥1\(\mu\)M in activated cells.

In response to antigen stimulation, IP\(_3\) is generated by the phospholipase C\(\gamma\) (PLC\(\gamma\))-mediated hydrolysis of the membrane glycerophospholipid, phosphatidylinositol 4,5-bisphosphate (PI(4,5)P\(_2\)). IP\(_3\) directly binds to and activates Ca\(^{2+}\) efflux through IP\(_3\) receptors. Ca\(^{2+}\) release from the ER is controlled by agents that regulate PLC\(\gamma\) activity. These include phosphatidylinositol 3,4,5-trisphosphate (PI(3,4,5)P\(_3\)) and Bruton’s tyrosine kinase (Btk) that help recruit PLC\(\gamma\) to the plasma membrane. Addition of wortmannin, an inhibitor of PI3kinase, or expression of PI(3,4,5)P\(_3\)-depleting phosphatases reduce Ca\(^{2+}\) release from stores due to suppression of IP\(_3\) production [25, 29]. Rho family GTPases also regulate this process [13]. A defect in IP\(_3\) production and Ca\(^{2+}\) release from a mutagenized variant of RBL-2H3 mast cells, termed B6A4C1 (C1) cells, could be rescued by the expression of activated forms of Rac and Cdc42, suggesting a potential role for these GTPases in PLC\(\gamma\) activation [9, 14]. Another factor that regulates Ca\(^{2+}\) release from the ER is the availability of the substrate for PLC\(\gamma\), PI(4,5)P\(_2\). The type I family of phosphatidylinositol 4-
phosphate 5-kinases (PIP5kinase-I) synthesize PI(4,5)P$_2$ by phosphorylation of a phosphatidylinositol 4-phosphate substrate. This family comprises of three isoforms: $\alpha$, $\beta$ and $\gamma$, including three splice variants of the $\gamma$ isoform ($\gamma^{87}$, $\gamma^{90}$ and $\gamma^{93}$) [15]. In HeLa cells, Wang et al. [37] identified PI(4,5)P$_2$ synthesized by PIP5kinase-I$\gamma^{87}$ as the source for PLC$\beta$-mediated IP$_3$ production. The kinase isoform responsible for the synthesis of the PLC$\gamma$ hydrolyzable pool of PI(4,5)P$_2$ in mast cells has not been identified yet.

The concept of Ca$^{2+}$ release activated currents ($I_{CRAC}$) was first proposed over two decades ago [24]. These currents describe the influx of Ca$^{2+}$ from the extracellular medium in response to depletion of ER Ca$^{2+}$ stores. Recently, two proteins involved in $I_{CRAC}$ have been identified. The stromal interaction molecule, STIM1, resides in the ER, where its luminal EF-hand motif detects Ca$^{2+}$ depletion from stores. This results in the activation of STIM1 to form puncta that translocate to regions close to the plasma membrane. Here, STIM1 interacts with the pore forming subunits of CRAC, termed Orai1, by a mechanism potentially involving their coiled coil domains. Orai1 subunits oligomerize to form the pore for the entry of Ca$^{2+}$. However, there are other channels that contribute to $I_{CRAC}$ which have not been definitively identified. It is known that entry of Sr$^{2+}$, and potentially other divalent cations, can trigger degranulation in RBL-2H3 mast cells [11]. In a recent study, Ma et al. [16] identified TRPC5, a transient receptor potential channel isoform, in mediating store operated Ca$^{2+}$ and Sr$^{2+}$ influx in mast cells and suggested that this channel may be acting in a heteromeric complex with Orai1 to potentiate Ca$^{2+}$ influx. Another isoform, TRPC1, was also proposed to regulate this process, albeit, less potently. Vennekens et al. [36] used TRPM4 channel knockout mice to show that this channel negatively regulates Ca$^{2+}$ influx in mast cells. Ca$^{2+}$ release
from stores was comparable in wt and TRPM4−/− mice, however, the influx phase was enhanced in the TRPM4−/− mice compared to wt mice. Dellis et al. [6] also reported a CRAC-independent influx of Ca2+ mediated by IP3R in the plasma membrane of B cells. However, such plasma membrane IP3R have not been reported in any other system.

Given the importance of Ca2+ mobilization in the capacity of mast cells to respond to antigenic substances, there are still gaps in our knowledge of how this response is modulated, especially with respect to the role of phosphoinositides. Recently, PIP5kinase-Iα was shown to negatively regulate the overall Ca2+ response in mast cells [27]. However, the mechanism of the negative regulation, and roles for other PIP5kinase-I isoforms in this process were not characterized. Moreover, the PIP5kinase-I isoform(s) that generates the PLCγ-hydrolyzable pool of PI(4,5)P2 in mast cells is still not known. In this study, we systematically investigated the roles for PIP5kinase-I isoforms in mast cell signaling, and found that the alpha and gamma isoforms regulate distinct stages of the mast cell Ca2+ response. We show by overexpression experiments that PIP5kinase-Iα is not important for IP3-mediated Ca2+ release from the ER. Moreover, we show for the first time that the PI(4,5)P2 produced by PIP5kinase-Iα negatively regulates the store-operated influx of Ca2+. We also demonstrate that PIP5kinase-Iγ contributes positively to IP3-mediated Ca2+ release from the ER. Further, we provide evidence that PIP5kinase-Iα and -Iγ synthesize functionally and spatially distinct pools of PI(4,5)P2 in the mast cell plasma membrane.
2.3 Materials and Methods

2.3.1 Materials.

Anti-DNP IgE was purified as described previously [23]. Murine wt and mutant HA-tagged PIP5kinase-Iα constructs were from Dr. Christopher Carpenter (Harvard U). GFP-tagged and HA-tagged PIP5kinase-Iγ87 and -Iγ90 constructs from Dr. P. De Camilli (Yale U.) have been described previously [8]. ECFP-Inp51 and DsRed PLCδ-PH domain were from Dr. A. Jeromin (Allan Institute of Brain Research, Seattle WA). L10-GFP, L10-Inp54-GFP, S15-GFP and S15-Inp54-GFP [26] were a gift from Dr. William Rodgers (Oklahoma Biomedical Research Foundation, OK), where L10 represents the lipid raft localized membrane anchoring domain of Lck (MGCVCSSNPE), and S15 represents the non-lipid raft localized membrane anchoring domain of c-Src (MGSSKSKPKDPSQRR).

2.3.2 Cell culture and generation of stable cell lines.

RBL-2H3 cells were cultured as monolayers in Minimum Essential Medium (Invitrogen Corp., Carlsbad, CA) with 20% Fetal Bovine Serum (Atlanta Biologicals, GA) and gentamycin (Invitrogen). Murine HA-tagged wt and catalytically inactive mutant (D203A) PIP5kinase-Iα constructs were cloned into a pIRES2-EGFP vector (Invitrogen Corp.) using XhoI/SacII restriction sites. Cells were transfected using Lipofectamine2000 in OptiMEM (Invitrogen Corp.) reduced serum medium. Cells were incubated with the DNA-lipofectamine complexes for 1hr, following which 0.1µM phorbol 1,2-dibutyrate (Sigma, St. Louis, MO) in OptiMEM was added to the cells for 4 hours to enhance fluid phase pinocy-
tosis and DNA uptake. Transfected cells were selected in MEM containing 380\(\mu\)g/mL neomycin (G418). Cells positive for PIP5kinase-I\(\alpha\) were identified based on EGFP expression. Three WT clones (I\(\alpha\)WT) and two mutant clones (I\(\alpha\)Mut) were produced and characterized.

ECFP-tagged wt yeast inositol polyphosphate 5-phosphatase, Inp51 [30], was stably transfected into RBL-2H3 cells using lipofectamine as described above. Cells positive for Inp51 were identified based on ECFP expression. Two clones (Inp) were produced and characterized.

PIP5kinase-I\(\gamma^{-/-}\) mice have been described previously [7]. Hematopoetic cells were extracted by flushing the bone marrow from legs of new born mice (see Appendix B for protocol). Cells were maintained in Dulbecco’s Minimum Essential Medium (DMEM) with 10% fetal bovine serum, and 20ng/ml each of IL-3 and stem cell factor (Peprotech Inc., NJ). After about 2 weeks in culture, differentiation into mast cells was confirmed by labeling surface receptors with Alexa488-anti-DNP IgE. The use of stem cell factor was discontinued at this point. The cells were maintained in suspension culture.

2.3.3 Immunocytochemistry.

Cells were plated at a subconfluent density of 0.5 \(\times\) 10\(^6\)/mL in 35mm MatTek dishes (MatTek Corporation, Ashland, MA) and cultured overnight. Cells were fixed the next day using 3.7% formaldehyde, permeabilized with 0.1% tritonX-100, and labeled for 1 hour with appropriate antibodies in the presence of 10mg/mL BSA. Images were collected using a Leica TCS SP2 laser scanning confocal system (Leica Microsystems Inc., Exton, PA) with a 63x, 0.9 nu-
merical aperture, HCX APO L U-V-I water immersion objective. For detecting HA-tagged wt and mutant PIP5kinase-Iα, anti-HA mAb (1:100; Covance) was used as the primary antibody, followed by Alexa568-anti mouse IgG1 as the secondary antibody (1:100; Invitrogen). FITC-Phalloidin (1:200; Invitrogen) was used to label membrane ruffles.

### 2.3.4 Immunoprecipitation.

PIP5kinase-Iα was immunoprecipitated using polyclonal anti-HA (Covance). Briefly, cells were resuspended in tyrodes-BSA at a concentration of $6.2 \times 10^6$/mL. 500 µL of 3X solubilization buffer (15mM N-ethylmaleimide, 3mM Na$_3$VO$_4$, 15mM Na$_2$P$_2$O$_7$, 10mM NaF, 6mM iodoacetate, 15mM EDTA, 0.3% TritonX-100, 150mM NaCl, and 150mM Tris) was added to 1mL cells and allowed to incubate on ice for 5min. Cell debris was pelleted by centrifuging at 14000 rpm at 4°C for 10min. 130 µL supernatant was incubated with 25 µL Protein A beads (Amersham, Piscataway, NJ), and 10 µL polyclonal anti-HA antibody for 2hrs at 4°C on a rotator. Beads were washed with 1X solubilization buffer three times to remove unbound antibody by pelleting the beads, discarding the supernatant, and resuspending in 500 µL of wash buffer. Finally, beads were boiled in 40 µL 2X sample buffer (Invitrogen). For polyacrylamide gel electrophoresis, 40 µL sample was loaded per lane of a 12% Tris-Glycine gel (Invitrogen) and run for about 1 hr at 35mA to resolve the bands. Blots were detected using the 10 µL 1mg/mL anti-HA mAb (Covance), 2 µL 0.5mg/mL biotinSP-goat anti mouse IgG (Jackson Immunoresearch, West Grove, PA), and 5 µL 2mg/mL NeutrAvidin-HRP (Pierce, Rockford, IL) in the presence of a blocking buffer (5 mL fish gelatin, 10 mL goat serum, 5 g BSA, 50 mL 10X Tris buffer, 0.5 mL tween,
and 434.5mL distilled water). Pico Enhanced ChemiLuminescence kit was used to develop the bands. GFP expression was confirmed by immunoprecipitating and detecting using an anti-GFP mAb (Invitrogen).

2.3.5 Measurement of PI(4,5)P$_2$ levels.

DsRED PLC$\delta$-PH domain was transiently transfected into the cells using either lipofectamine or electroporation, and used as a marker for PI(4,5)P$_2$ [34]. For electroporation, cells were harvested and resuspended at $5 \times 10^6$/ml in an electroporation buffer (137mM NaCl, 2.7mM KCl, 1mM MgCl$_2$, 1mg/mL glucose, 20mM HEPES, pH=7.4). 500$\mu$l cells premixed with 6$\mu$g DNA were pulsed once (exponential decay, 280V, 90$\mu$F) in a Bio-Rad Gene Pulser (Bio-Rad Laboratories, Hercules, CA). Cells were resuspended in media immediately and plated at $0.5 \times 10^6$/mL in MatTek dishes and cultured overnight. PI(4,5)P$_2$ levels were measured using real time confocal microscopy as previously described [5]. Briefly, the plasma membrane was labeled with 2$\mu$g/mL Cy5-cholera toxin for 4 min prior to imaging. Images were then collected every 3 sec for a total of 3 min in the absence of any stimulation using a Leica confocal TCS SP2 microscope. 0.5$\mu$M Ca$^{2+}$ ionophore or 0.8$\mu$g/mL DNP-BSA was added and images were collected every 3 sec for a total of 5 min post-stimulation. ImageJ$^1$ and MATLAB (The MathWorks, Inc., MA) were used for quantitative image analysis as shown in Figure 2.1 [5]. Briefly, a region of interest enclosing the selected cell was chosen in ImageJ. Matlab was used to generate a PM mask by thresholding the CtxB image. Extraneous pixels were removed via a binning operation to generate a final mask. The PLC$\delta$-PH fluorescence was calculated as the mean

$^1$http://rsb.info.nih.gov/ij/
intensity of all pixels in the PM mask, before and after stimulation.

2.3.6 \textbf{Ca}^{2+} \textbf{measurements.}

Intracellular Ca\textsuperscript{2+} levels were measured using either an SLM 8100C steady state fluorimeter (SLM instruments, Urbana, IL) or a flow cytometer (BD LSR II, Becton Deckinson Biosciences, NJ). For fluorimetry, cells in suspension were loaded with the Ca\textsuperscript{2+} indicator, indo-1 (Invitrogen Corp., ex=330nm, em=400nm), and sensitized with anti-DNP IgE in Tyrodes buffer containing 0.5mM sulfinpyrazone (Sigma). Cells were stimulated with 0.2\,µg/mL DNP-BSA, or 0.25\,µM thapsigargin, and their Ca\textsuperscript{2+} response monitored for about 500sec. The cells were then lysed by the addition of 0.1\% TritonX-100 to obtain the maximal value of indo-1 fluorescence for that sample. The fluorescence was then quenched by the addition of EDTA to obtain the background indo-1 fluorescence levels. Representative Ca\textsuperscript{2+} responses were plotted as the change in fluorescence intensity of indo-1 with time, where the fluorescence intensity was expressed as the ratio of the actual fluorescence value and the difference between the TritonX-100 and EDTA treated fluorescence values. Ca\textsuperscript{2+} responses in the presence of extracellular Ca\textsuperscript{2+} were integrated for 300-500 sec. Responses were quantified as a ratio of the integrated area of the Ca\textsuperscript{2+} response for the sample to that of untransfected 2H3 cells.

For flow cytometry, fura-red (Invitrogen Corp.) was used as a Ca\textsuperscript{2+} indicator and loaded at a concentration of 0.5\,µM as described for indo-1. Ca\textsuperscript{2+} ionophore was added to obtain the maximal response. Representative Ca\textsuperscript{2+} responses were plotted as the inverse of the change in average intensity of fura-red with time,
STEP 1: Obtain a PM trace using the Cy5-CtxB label

STEP 2: Threshold original image

STEP 3: Select ROI containing a single cell and apply a median filter to generate the final mask

STEP 4: Use PM mask to determine mean intensity of DsRED-PLCδ PH domain at the PM for each cell

Figure 2.1: Quantitative image analysis of DsRED-PLCδ PH domain recruitment to the plasma membrane. The PM was labeled with 2µg/mL Cy5-Cholera toxin B for 4 min prior to imaging. Images were collected every 3 sec for 3 min prior to stimulation. 0.5µM Ca^{2+} ionophore or 0.8µg/mL antigen was then added and images were collected every 3 sec for 5 min post stimulation. Matlab was used for quantitative image analysis.
where the fluorescence intensity was expressed as the ratio of the average fluorescence value and the difference between the ionophore-treated and baseline fluorescence values. Responses were quantified as a ratio of the integrated area of the Ca\(^{2+}\) response for the sample to that of 2H3 cells.

### 2.3.7 Ruffling assay.

Cells were plated at a subconfluent density of \(0.5 \times 10^6/\text{mL}\) in 35mm Mat-Tek dishes and sensitized with anti-DNP IgE overnight. Cells were stimulated with 0.8\(\mu\)g/mL DNP-BSA for 15 min at 37\(^\circ\)C, fixed using 3.7% formaldehyde, permeabilized with 0.1% tritonX-100, and labeled with either FITC or TRITC-conjugated phalloidin (Invitrogen Corp.) to visualize the ruffles. Cells expressing the cDNA were scored based on their morphology as being ruffled or not ruffled (see Results).

### 2.4 Results

#### 2.4.1 Ectopically expressed PIP5kinase-I\(\alpha\) synthesizes a plasma membrane pool of PI(4,5)P\(_2\) in RBL-2H3 cells.

To investigate the role of PI(4,5)P\(_2\) synthesis by PIP5kinase-I\(\alpha\) in IgE receptor signaling in RBL-2H3 mast cells, we stably expressed murine HA-tagged wt PIP5kinase-I\(\alpha\) and a catalytically inactive (D203A) mutant of the kinase [33]. Three wt clones (I\(\alpha\)WT) and two mutant clones (I\(\alpha\)Mut) were characterized and
their phenotypes compared with untransfected RBL-2H3 cells (2H3). Figure 2.2 shows representative images for EGFP expression from the pIRES2-EGFP vector and kinase localization in individual IαWT and IαMut clones. When labeled by an anti-HA mAb that detects the kinase, both wt and mutant PIP5kinase-Iα show relatively low expression levels with some localization to the plasma membrane and in cytoplasmic structures. 2H3 cells did not show EGFP fluorescence or significant labeling by the anti-HA mAb. Expression was confirmed by western blotting: Wt and mutant PIP5kinase-Iα were immunoprecipitated from individual IαWT and IαMut clones, respectively, and detected using anti-HA antibodies, with 2H3 cells as a negative control (Figure 2.3).

To evaluate the effects of IαWT and IαMut on PI(4,5)P₂ levels at the plasma membrane, the PI(4,5)P₂-specific DsRED PLCδ-PH domain was used to quantitatively monitor plasma membrane PI(4,5)P₂ using real time confocal microscopy and a plasma membrane masking algorithm as described in Methods. Similar, strong plasma membrane localization of the labeled PH domain was observed in 2H3, IαWT and IαMut cells, with no significant differences detected in the ratio of plasma membrane to cytoplasmic DsRED PLCδ-PH for these cells under resting conditions (Figure 2.4). As shown in Figure 2.5, stimulation of 2H3 cells with multivalent antigen does not cause a significant change in average PI(4,5)P₂ levels at the plasma membrane as monitored over 300 sec with DsRED PLCδ-PH. Although antigen-stimulated PI(4,5)P₂ hydrolysis is expected to reduce the plasma membrane PI(4,5)P₂ levels, it is apparently compensated by stimulated PI(4,5)P₂ synthesis on this timescale [2]. Also shown in Figure 2.5, only small differences in the antigen-stimulated changes in PI(4,5)P₂ levels were observed for the IαWT and IαMut cells compared to 2H3 cells.
Figure 2.2: Expression and subcellular localization of HA-tagged wt PIP5kinase-\(\mathrm{I}_\alpha\) and catalytically inactive mutant kinase by immunocytochemistry. Cells expressing the kinase isoforms were identified based on EGFP expression via an IRES sequence in the vector. The HA-tag was labeled in fixed cells with anti-HA mAb (1:100) and Alexa568 anti-mouse IgG1 (1:100). Control untransfected RBL-2H3 cells show no EGFP expression and only background Alexa568 label.
Figure 2.3: Expression of HA-tagged WT PIP5kinase-Iα and catalytically inactive mutant kinase by western blotting. Wt and mutant PIP5kinase-Iα were immunoprecipitated from individual IαWT and IαMut clones, respectively, using a rabbit polyclonal anti-HA antibody. Anti-HA mAb was used for the detection of blots. EGFP expression was confirmed by immunoprecipitating and detecting using an anti-GFP antibody.
Similar to antigen stimulation, no significant changes in plasma membrane PI(4,5)P\textsubscript{2} levels were detected following stimulation with the Ca\textsuperscript{2+} ionophore, A23187 in 2H3 cells. However, A23187 stimulated an increase in the plasma membrane recruitment of the PH domain in I\alpha WT cells (Figure 2.6), suggesting a net increase in stimulated PI(4,5)P\textsubscript{2} synthesis at the plasma membrane by wtPIP5kinase-I\alpha. In contrast, I\alpha Mut cells exhibited an ionophore-stimulated decrease in plasma membrane PI(4,5)P\textsubscript{2} levels, suggesting possible dominant negative inhibition of the endogenous kinase by this mutant coupled with some PI(4,5)P\textsubscript{2} hydrolysis (Figure 2.6). It is not clear why a similar dominant negative effect of mutant PIP5kinase-I\alpha is not observed in antigen-stimulated cells, but it is possible that antigen stimulates additional pathways of PI(4,5)P\textsubscript{2} synthesis that might compensate for this effect (see below).

We also examined the capacity of these cells to ruffle in response to antigen stimulation [21]. As shown in Figure 2.7, I\alpha Mut cells exhibit greatly suppressed ruffling at the dorsal cell surface relative to either 2H3 or I\alpha WT cells. Results from multiple experiments are summarized in Figure 2.8. They show that stimulated ruffling in I\alpha Mut cells is suppressed by \(~80\%\) compared to 2H3 or I\alpha WT cells, which show similarly strong ruffling responses. These results are consistent with the characteristics of the PIP5kinase-I\alpha\textsuperscript{−/−} BMMCs, which showed reduced F-actin content before and after antigen stimulation [27]. They further support the conclusion above that mutant PIP5kinase-I\alpha can act as a dominant negative suppressor of endogenous PIP5kinase-I\alpha activity in stimulated cells.
Figure 2.4: Expression of transiently transfected DsRED-PLCδ-PH domain in 2H3 cells by confocal microscopy. The PLCδ-PH domain was used as indicator for PI(4,5)P_2 at the plasma membrane.
Figure 2.5: Net PI(4,5)P2 levels at the plasma membrane before and after stimulation with antigen (0.8 µg/mL). Plots represent average DsRED-PLCδ PH domain intensity at the plasma membrane of 6 cells each for IαWT or IαMut clone, and for untransfected 2H3 cells.
Figure 2.6: Net increase in PI(4,5)P$_2$ levels at the plasma membrane before and after stimulation with Ca$^{2+}$ ionophore, A23187 (0.5µM). Plots represent average DsRED-PLCδ PH domain intensity at the plasma membrane of 6 cells each for IαWT or IαMut clone, and for untransfected 2H3 cells.
Figure 2.7: Expression of mutant PIP5kinase-Iα suppresses stimulated cell ruffling. 2H3, IαWT and IαMut cells were sensitized with anti-DNP mouse IgE and either fixed without stimulation (-Ag) or stimulated (+Ag) for 15 min with 0.8 µg/mL DNP-BSA at 37°C. Membrane ruffles were labeled with FITC-phallolidin (1:200) post fixation. Images for -Ag show equatorial sections; +Ag images show dorsal surfaces. Images were taken using a Leica SP2 TCS confocal microscope and scale bars show 20µm.
Figure 2.8: **Quantitation of stimulated ruffling for PIP5kinase-Iα overexpression.** More than 600 cells per sample from two independent experiments were scored based on morphology of phalloidin-labeled cells. Bars represent percentage of cells that show antigen-stimulated ruffling for 2H3, IαWT and IαMut cells. Error bars represent SEM. *, P < 0.05 for IαMut cells compared to 2H3 cells.
2.4.2 PIP5kinase-Iα negatively regulates store-operated Ca\(^{2+}\) influx.

To investigate the possible role of PIP5kinase-Iα in antigen-stimulated Ca\(^{2+}\) mobilization in RBL cells, we measured Ca\(^{2+}\) levels in suspended 2H3, IαWT and IαMut cells using the Ca\(^{2+}\) indicator indo-1 in steady-state fluorimetry. As shown in Figure 2.9, antigen stimulates a biphasic Ca\(^{2+}\) response that is somewhat larger and faster for the IαMut cells than for the 2H3 cells. In contrast, the IαWT cells exhibit a rapid initial response but a clearly attenuated plateau phase compared to the 2H3 cells. When multiple experiments are averaged, the IαWT cells exhibit a time-integrated response that is about 50% of the 2H3 cell response, whereas the IαMut cells exhibit about a 10% larger integrated response than the parental 2H3 cells (Figure 2.12). These results are consistent with those reported for antigen-stimulated BMMCs from PIP5kinase-Iα knockout mice, in which the absence of PIP5kinase-Iα resulted in a ∼20% larger sustained plateau phase of the Ca\(^{2+}\) response than that for wt BMMCs [27].

To determine whether PIP5Kinase-Iα contributes to the pool of PI(4,5)P\(_2\) that is hydrolyzed by antigen-stimulated PLC\(\gamma\) to produce IP\(_3\), we examined the Ca\(^{2+}\) response to antigen in the absence of extracellular Ca\(^{2+}\). Under these conditions, the transient response observed is completely dependent on IP\(_3\)-mediated Ca\(^{2+}\) release from ER stores [37]. As shown in Figure 2.10, 2H3, IαWT and IαMut cells all showed similar, transient responses under these conditions, and these integrated responses from multiple experiments are not significantly different from each other (Figure 2.12). These results suggest that PIP5Kinase-Iα is not the primary enzyme responsible for the pool of PI(4,5)P\(_2\) that is hydrolyzed by PLC\(\gamma\) in these cells.
To further investigate the role of PIP5kinase-Iα in regulating Ca\(^{2+}\) influx in RBL mast cells, we by-passed early signaling by FcεRI and activated store-operated Ca\(^{2+}\) influx using thapsigargin to inhibit the sarcoplasmic/endoplasmic reticulum Ca\(^{2+}\) ATPase (SERCA). As shown in Figure 2.11, low concentrations of thapsigargin cause a rapid increase in cytoplasmic Ca\(^{2+}\) levels in these cells, followed by a sustained phase that is due to store operated Ca\(^{2+}\) influx ([1]; data not shown). Iα WT cells showed a response to thapsigargin that is ∼45% less than that for 2H3 cells, whereas the response to thapsigargin in the Iα Mut cells was not significantly different from that for 2H3 cells (Figure 2.12). These results indicate that negative regulatory effect of Iα WT overexpression observed for antigen-stimulated Ca\(^{2+}\) mobilization is due primarily to the negative regulation of store-operated Ca\(^{2+}\) influx.

### 2.4.3 Plasma membrane-associated yeast inositol polyphosphate 5′-phosphatase, Inp51, alters stimulated Ca\(^{2+}\) responses

To further investigate the roles of PI(4,5)P\(_2\) at the plasma membrane in FcεRI-mediated Ca\(^{2+}\) responses, we stably expressed an ECFP-tagged yeast inositol polyphosphate 5′-phosphatase, Inp51 (Inp cells) [30] that dephosphorylates PI(4,5)P\(_2\) to generate PI4P. As shown in Figure 2.13, Inp51 exhibits strong plasma membrane association, as well as some cytoplasmic localization. These Inp-expressing cells did not show significant differences in the ratio of DsRED-PLCδ-PH domain at the plasma membrane to that in the cytoplasm when this PI(4,5)P\(_2\) reporter was co-expressed, indicating that expression of the Inp phospho-
Figure 2.9: $\text{Ca}^{2+}$ response to antigen stimulation for 2H3, I\text{WT} and I\text{Mut} cells. Traces show representative $\text{Ca}^{2+}$ responses for 2H3, I\text{WT} and I\text{Mut} cells stimulated with 0.2$\mu$g/mL antigen from one experiment.
Figure 2.10: Ca$^{2+}$ response to antigen stimulation in the absence of extracellular Ca$^{2+}$ for 2H3, IαWT and IαMut cells. Traces show representative Ca$^{2+}$ responses for 2H3, IαWT and IαMut cells stimulated with 0.2µg/mL antigen in the absence of extracellular Ca$^{2+}$ from one experiment.
Figure 2.11: Ca^{2+} response to stimulation by thapsigargin for 2H3, IαWT and IαMut cells. Traces show representative Ca^{2+} responses for 2H3, IαWT and IαMut cells stimulated with 0.25µM thapsigargin from one experiment.
Figure 2.12: Quantitation of Ca$^{2+}$ response for 2H3, IαWT and IαMut cells. Ca$^{2+}$ responses in the presence of extracellular Ca$^{2+}$ were integrated for 500 sec, and results from six independent experiments were averaged. ***, $P < 0.001$ for IαWT cells compared to 2H3 cells in the presence of antigen. **, $P < 0.005$ for IαWT cells compared to 2H3 cells in the presence of thapsigargin. Ca$^{2+}$ responses in the absence of extracellular Ca$^{2+}$ were integrated for 300 sec, and results from five independent experiments were averaged. Bars represent responses relative to that for 2H3 cells, and error bars show SEM.
phatase did not detectably alter the steady state levels of PI(4,5)P$_2$ at the plasma membrane (data not shown). However, using this PI(4,5)P$_2$ probe, we observed the loss of apparent thapsigargin-stimulated synthesis of PI(4,5)P$_2$ at the plasma membrane in Inp cells compared to 2H3 cells (Figure 2.14). The antigen-stimulated levels of PI(4,5)P$_2$ were not monitored.

To investigate whether a reduction in stimulated PI(4,5)P$_2$ levels by Inp51 could alter Ca$^{2+}$ responses to FcεRI crosslinking, we compared the antigen-stimulated Ca$^{2+}$ response in Inp cells to that in 2H3 cells. As shown in Figure 2.15, the antigen-stimulated Ca$^{2+}$ response is enhanced by $\sim$20% in Inp cells. This enhancement is somewhat greater for the sustained phase of thapsigargin-stimulated cells ($\sim$30%; Figure 2.16), indicating a principle effect on store-operated Ca$^{2+}$ influx. In contrast, antigen-stimulated Ca$^{2+}$ release from stores, monitored in the absence of extracellular Ca$^{2+}$, was reduced by $\sim$40% in Inp cells (Figure 2.17). These results are summarized for integrated responses from multiple experiments in Figure 2.18. They support the findings with PIP5kinase-I$\alpha$ overexpression for a negative regulatory effect of PI(4,5)P$_2$ at the plasma membrane on store operated Ca$^{2+}$ influx. Furthermore, they also implicate PI(4,5)P$_2$ at the plasma membrane in antigen-stimulated Ca$^{2+}$ release from stores, most likely as the source of IP$_3$ generated by activated PLC$\gamma$.

### 2.4.4 PIP5kinase-I$\gamma$ contributes to antigen-mediated Ca$^{2+}$ release from stores.

The enhanced Ca$^{2+}$ response to antigen observed in PIP5kinase-I$\alpha^{-/-}$ BMMC mast cells [27], and the lack of effect of mutant or wt PIP5kinase-I$\alpha$ on anti-
Figure 2.13: **Expression and subcellular localization of ECFP-Inp51 compared to untransfected 2H3 cells by confocal microscopy.** Representative images for expression of Inp51 are shown in 2H3 and Inp cells. ECFP-Inp51 shows plasma membrane localization in Inp cells. No ECFP fluorescence is detected for 2H3 cells. Images were collected using a Leica SP2 TCS confocal microscope.
Figure 2.14: Net decrease in PI(4,5)P$_2$ levels at the PM in cells overexpressing Inp51 observed upon stimulation with thapsigargin. Cells were stimulated with 0.25µM thapsigargin. Plots represent average DsRED-PLCδ PH domain intensity at the plasma membrane of 4 cells for Inp relative to that for 2H3 cells.
Figure 2.15: Enhanced Ca\textsuperscript{2+} response to antigen for Inp cells compared to 2H3 cells. Traces show representative total Ca\textsuperscript{2+} response for 2H3 (grey) and Inp (blue) cells stimulated with 0.2\(\mu\text{g}/\text{mL}\) antigen from one experiment.
Figure 2.16: Enhanced Ca\textsuperscript{2+} response to thapsigargin for Inp cells compared to 2H3 cells. Traces show representative Ca\textsuperscript{2+} response to thapsigargin for 2H3 (grey) and Inp (blue) cells stimulated with 0.25 µM thapsigargin from one experiment.
Figure 2.17: Decreased antigen-stimulated Ca\textsuperscript{2+} release from stores for Inp cells compared to 2H3 cells. Traces show representative Ca\textsuperscript{2+} response in the absence of extracellular Ca\textsuperscript{2+} for 2H3 (grey) and Inp (blue) cells stimulated with 0.2µg/mL antigen from one experiment.
Figure 2.18: Quantitation of Ca$^{2+}$ response for Inp and 2H3 cells. Ca$^{2+}$ responses in the presence of extracellular Ca$^{2+}$ were integrated for 500 sec, and results from three independent experiments were averaged. Bars represent responses relative to 2H3 cells, and error bars show SEM. **, $P < 0.005$ for Inp cells compared to 2H3 cells in the presence of antigen. Ca$^{2+}$ responses in the absence of extracellular Ca$^{2+}$ were integrated for 300 sec, and results from three independent experiments were averaged. *, $P < 0.05$ for Inp cells compared to 2H3 cells.
gen stimulated release from stores in RBL mast cells (Figure 2.12) indicate that this isoform does not contribute to the pool of PI(4,5)P₂ that is hydrolyzed by PLCγ in response to FcεRI activation. A previous study demonstrated that PIP5kinase-Iγ (but not PIP5kinase-Iα) is important for the pool of PI(4,5)P₂ that is hydrolyzed by PLCβ in response to G protein-coupled receptor activation in HeLa cells [37]. To evaluate the role of PIP5kinase-Iγ in FcεRI-mediated Ca²⁺ mobilization, we examined this response in BMMCs from wt and PIP5kinase-Iγ⁻/⁻ mice [7]. BMMCs were differentiated from newborn wt and PIP5kinase-Iγ⁻/⁻ mice using IL3 and SCF, and similar surface expression of FcεRI on these cells was confirmed using Alexa488-anti-DNP IgE (Figure 2.19).

As shown in Figure 2.20, both antigen-stimulated Ca²⁺ release from stores and antigen-stimulated Ca²⁺ influx are substantially reduced in the absence of PIP5kinase-Iγ. As summarized for multiple experiments in Figure 2.21, the integrated Ca²⁺ response to antigen in the presence of extracellular Ca²⁺ was reduced by ~40% in the absence of PIP5kinase-Iγ, and the response to antigen in the absence of extracellular Ca²⁺ was reduced by 50%. These results indicate that PIP5kinase-Iγ contributes to FcεRI-mediated Ca²⁺ mobilization, most likely by synthesizing the pool of PI(4,5)P₂ that is hydrolyzed by PLCγ to produce IP₃. Because both PIP5kinase-Iγ87 and PIP5kinase-Iγ90 splice variants and the catalytically inactive mutants of these enzymes strongly localize to the plasma membrane of RBL mast cells (Figure 2.22), this isoform of PIP5kinase-Iγ is likely to synthesize PI(4,5)P₂ at the plasma membrane, similar to the PIP5kinase-Iα isoform as discussed above.
Figure 2.19: FceRI expression on WT and PIP5kinase-Iγ−/− BMMCs. FceRI expression was used as an indication of differentiation into mast cells, and detected by labeling BMMCs with Alexa488-anti-DNP mouse IgE for 1 hr at 37°C. Images were collected using a Leica SP2 TCS confocal microscope.
Figure 2.20: Antigen-stimulated Ca$^{2+}$ response for WT and PIP5kinase-I$\gamma^{-/-}$ BMMCs. Plots show representative Ca$^{2+}$ response from one experiment. The Ca$^{2+}$ response was first triggered in the absence of extracellular Ca$^{2+}$ by low dose of antigen (0.1$\mu$g/mL) and release from stores was monitored for 300 sec. Extracellular Ca$^{2+}$ was restored by the addition of 2mM CaCl$_2$, and Ca$^{2+}$ influx was monitored for 250 sec.
Figure 2.21: Quantitation of the Ca\textsuperscript{2+} response for WT and PIP5kinase-I\textsubscript{γ−/−} BMMCs. Ca\textsuperscript{2+} responses were integrated for 500 sec. Bars represent responses normalized to WT BMMCs and error bars show SEM for data from six experiments in the presence of extracellular Ca\textsuperscript{2+} and four experiments in the absence of extracellular Ca\textsuperscript{2+}. **, P < 0.005 for KO BMMCs compared to WT BMMCs.
Figure 2.22: Transient expression of WT and mutant PIP5kinase-\( \gamma87 \) and -\( \gamma90 \) in 2H3 cells. EGFP-tagged WT and K188A mutant PIP5kinase-\( \gamma \) were visualized directly in fixed cells. Expression of HA-tagged WT PIP5kinase-\( \gamma \) was detected in fixed cells by permeabilizing with 0.1% tritonX-100, and labeling with anti-HA mAb followed by Alexa488-anti mouse IgG\(_1\) antibody. Images were collected using a Leica SP2 TCS confocal microscope and scale bars show 20\( \mu \)m.
2.4.5 Lipid raft-targeted depletion of PI(4,5)P$_2$ enhances Ca$^{2+}$ responses to antigen

Our results suggest that the pools of PI(4,5)P$_2$ synthesized by PIP5kinase-I$\alpha$ and -I$\gamma$ are functionally segregated at the plasma membrane. Previous studies have suggested the association of receptor-coupled PI(4,5)P$_2$ pools with lipid rafts [17, 22]. To investigate if one of these PI(4,5)P$_2$ pools observed in the regulation of Ca$^{2+}$ mobilization was preferentially associated with lipid rafts, we targeted the PI(4,5)P$_2$-specific phosphatase, Inp54, to rafts using the plasma membrane anchoring sequence of Lck (L10-Inp54-GFP) [26]. The phosphatase was also targeted to non-raft regions using the plasma membrane anchoring sequence of Src (S15-Inp54-GFP), that is excluded from lipid rafts [26]. Figure 2.23 shows representative images for the localization of these constructs in RBL-2H3 cells. L10-GFP and S15-GFP were used as a control. L10-GFP and L10-Inp54-GFP localized strongly to the plasma membrane. S15-GFP localized to the plasma membrane less efficiently and showed expression in the cytoplasm consistent with previous observations [4].

The overall Ca$^{2+}$ response of raft targeted Inp54 was measured using flow cytometry as described in Methods. Cells were transiently transfected and classified as transfected or untransfected based on the GFP expression. Figure 2.24 and 2.25 show the representative plots of the mean fura red fluorescence after stimulation for cells expressing L10-GFP and L10-Inp54-GFP respectively, compared to untransfected cells. Figure 2.26 shows the integrated Ca$^{2+}$ responses for untransfected cells and cells expressing L10-GFP and L10-Inp54-GFP from two independent experiments. The integrated Ca$^{2+}$ response for L10-GFP were reduced by 50% compared to untransfected cells. The mechanism by which
Figure 2.23: Expression and subcellular localization of raft and non-raft targeted Inp54 in RBL-2H3 cells. Representative images are shown for transiently transfected L10-GFP, L10-Inp54-GFP, S15-GFP and S15-Inp54-GFP in RBL-2H3 cells. Images were collected using a Leica SP2 TCS confocal microscope and scale bars show 25 µm.
targeted localization of GFP to ordered lipid microdomains reduces calcium influx is presently unclear. The integrated Ca\textsuperscript{2+} response for cells expressing L10-Inp54-GFP was 1.6 times that observed for untransfected cells. The Ca\textsuperscript{2+} responses for cells expressing S15-GFP and S15-Inp54-GFP were variable (data not shown). From the above data, we predict that calcium influx is regulated in ordered lipid microdomains at the plasma membrane. Ongoing efforts are focussed on understanding the mechanisms of this regulation.

2.5 Discussion

The presence of multiple independent signaling microdomains in cells has been long predicted to contribute to the diversity of cellular responses. Such a segregation of signaling pathways becomes useful when explaining the functional versatility of molecules such as PI(4,5)P\textsubscript{2}, wherein the same lipid species can turn-on or turn-off different processes in response to agonist stimulation. In this study, we employed overexpression and mouse knockout strategies to show that PIP5kinase-I\textsubscript{\alpha} and -I\textsubscript{\gamma} synthesize functionally distinct pools of PI(4,5)P\textsubscript{2} at the plasma membrane. The reduction in the initial phase of the Ca\textsuperscript{2+} response in PIP5kinase-I\textsubscript{\gamma\textsuperscript{−/−}} BMMCs suggests that PI(4,5)P\textsubscript{2} synthesized by PIP5kinase-I\textsubscript{\gamma} positively regulates Fc\textsubscript{\epsilon}RI-stimulated Ca\textsuperscript{2+} release from ER stores. Overexpression of wt PIP5kinase-I\textsubscript{\alpha} does not affect this initial step of Ca\textsuperscript{2+} release, but surprisingly, negatively regulates Ca\textsuperscript{2+} influx. In agreement with these observations, overexpression of a yeast inositol polyphosphate 5'-phosphatase, Inp51, reduces Ca\textsuperscript{2+} release from stores, and enhances Ca\textsuperscript{2+} influx, presumably due to depletion of plasma membrane pools of PI(4,5)P\textsubscript{2} generated by both PIP5kinase isoforms. In addition, preliminary data suggests that PIP5kinase-I\textsubscript{\alpha} may be
Figure 2.24: Ca$^{2+}$ response to antigen for cells expressing L10-GFP compared to untransfected cells. Traces show representative total Ca$^{2+}$ response to 0.4µg/mL antigen for 2H3 cells expressing (GFP pos; green) and not expressing (GFP neg; red) L10-GFP as measured by flow cytometry in one experiment.
Figure 2.25: Ca\textsuperscript{2+} response to antigen for cells expressing L10-Inp54-GFP compared to untransfected cells. Traces show representative total Ca\textsuperscript{2+} response to 0.4\(\mu\)g/mL antigen for 2H3 cells expressing (GFP pos; blue) and not expressing (GFP neg; red) L10-Inp54-GFP as measured by flow cytometry in one experiment.
Figure 2.26: Quantitation of total Ca\(^{2+}\) response for cells expressing L10-GFP and L10-Inp54-GFP. Ca\(^{2+}\) responses for cells expressing L10-GFP or L10-Inp54-GFP were integrated for 9 min, and results from two independent experiments were averaged. Bars represent responses relative to the integrated response for GFP negative cells, and error bars show SEM.
preferentially associated with lipid rafts. Expression of yeast inositol polyphosphate 5′-phosphatase, Inp54, recruited specifically to rafts by means of the membrane anchoring sequence of Lck enhances Ca²⁺ influx, and therefore, seems to affect the pool of PI(4,5)P₂ synthesized by PIP5kinase-Ⅰα.

**PI(4,5)P₂ generated by PIP5kinase-Ⅰγ functions as PLC substrate.**

Wang et al. [37] employed siRNA-mediated knockdown of PIP5kinase-Ⅰγ87 and -Ⅰγ90 to investigate their role in G-protein coupled receptor mediated Ca²⁺ signaling in HeLa cells. Knockdown of PIP5kinase-Ⅰγ87 reduced IP₃ production and the histamine stimulated Ca²⁺ response in these cells. The thapsigargin mediated response was unaffected suggesting that the -Ⅰγ87 isoform was responsible for supplying PI(4,5)P₂ as substrate to PLCβ. It is interesting to note that siRNA mediated knockdown of PIP5kinase-Ⅰα or -Ⅰβ did not affect PLCβ mediated IP₃ production and Ca²⁺ release from stores in HeLa cells, suggesting functional compartmentalization of PIP5kinase-I isoforms in these cells as well. This is also in agreement with the PIP5kinase-Ⅰα results where knockout of PIP5kinase-Ⅰα did not affect IP₃ production despite reduced levels of PI(4,5)P₂ [27]. Our results are in agreement with the above data suggesting that PIP5kinase-Ⅰγ but not PIP5kinase-Ⅰα regulates Ca²⁺ release from the ER.

**Mechanisms of regulation of Ca²⁺ influx by PI(4,5)P₂**

The reduction in Ca²⁺ influx in the presence of wt PIP5kinase-Ⅰα was most surprising to us. Such a negative regulatory role for this isoform was proposed in the study using PIP5kinase-Ⅰα⁻/⁻ mice [27]. However, this effect was attributed
to an enhancement of FcεRI activation. Although we observe a similar negative regulation of Ca^{2+} influx, we hypothesize that this outcome is independent of FcεRI activation and is potentially due to an effect of PI(4,5)P_2 production on the influx machinery itself. First, contrary to results from the PIP5kinase-Iα^{−/−} BMMCs, we find that PI(4,5)P_2 positively regulates FcεRI phosphorylation in RBL-2H3 cells (discussed in Chapter 3). Second, Ca^{2+} influx in the presence of PI(4,5)P_2 generated by wt PIP5kinase-Iα is diminished to both antigen and thapsigargin, suggesting an effect downstream of Ca^{2+} store depletion. Third, depletion of PI(4,5)P_2 by the expression of Inp51 diminishes Ca^{2+} release from stores but enhances Ca^{2+} influx, suggesting that these two processes are independently regulated. Based on related work reported in the literature, we hypothesize several candidates that potentially interact with PI(4,5)P_2 in this negative regulatory mechanism.

The transient receptor potential channel, TRPM4, also called Melastatin, is a very strong candidate as a negative regulator of Ca^{2+} influx. TRPM4 is a Ca^{2+} activated non selective cation channel, that when expressed in non-voltage gated cells depolarizes their membrane in response to elevated cytosolic Ca^{2+}. Previous studies have shown that depolarization of mast cells causes a reduction in the Ca^{2+} influx and degranulation responses [18]. PI(4,5)P_2 is a potent activator of TRPM4. Mice lacking TRPM4 show phenotypes strikingly similar to PIP5kinase-Iα^{−/−} mice [18]. In both cases, the mice are viable, show stronger allergic responses compared to wt mice, and have an enhanced Ca^{2+} response that is independent of release from stores [27, 36]. Nilius et al. [19] demonstrated that PI(4,5)P_2 exerts its effects on this channel via a PH consensus sequence in the C-terminus of TRPM4. They propose that binding of PI(4,5)P_2 to polybasic residues in this region of TRPM4 increases channel open probability.
They also propose an alternate mechanism wherein newly synthesized PI(4,5)P₂ competes with an inhibitory protein occupying the PH-consensus sequence and activates the channel. Hence, overexpression of PIP5kinase-Iα may depolarize the plasma membrane of RBL-2H3 cells by activating TRPM4 channels and thus, reduce Ca²⁺ influx.

TRPC channels are other members of the transient receptor potential channel family that act as non-selective Ca²⁺ permeable cation channels. Recently, Ma et al. demonstrated that several TRPC isoforms including TRPC 1, 2, 3, 5, 6 and 7 were expressed in RBL-2H3 mast cells [16]. ShRNA-mediated knock-down of TRPC5, and TRPC1 to a smaller extent, reduced the entry of Sr²⁺ and Ca²⁺ in these cells in response to thapsigargin, suggesting that these channels were activated in response to store depletion. In addition, this influx is significantly reduced upon knockdown of TRPC5 concurrently with Orai1 or STIM1. Further, Ma et al. showed that coexpression of STIM1 and Orai1 permitted Ca²⁺ influx but blocked Sr²⁺, whereas coexpression of STIM1 with TRPC5 permitted the influx of both Ca²⁺ and Sr²⁺. Reports from the literature suggest that TRPC channels are activated by the depletion of PI(4,5)P₂ by an unknown mechanism [35]. Hence, if TRPC5 functions in a multimeric complex with Orai1 and STIM1 as suggested by Ma et al., then increase in PI(4,5)P₂ levels can account for reduced activation of TRPC5, and hence, reduced Ca²⁺ influx.

The mediators of CRAC, Orai1 and STIM1, associate partly due to electrostatic interaction in their coiled coil domains. The C-terminus of STIM1 has polybasic residues, while the coiled coil region of Orai1 contains negatively charged residues. It is, therefore, possible that PI(4,5)P₂ may electrostatically compete with Orai1 for association with STIM1, or may affect the oligomeriza-
tion of Orai1 subunits by repulsion of their negative charges. Recent studies in the Baird-Holowka lab show that PI(4,5)P₂ in fact positively regulates the interaction between CRAC and Orai1 and the fluorescence energy transfer between these labeled subunits is enhanced when wt PIP5kinase-Iα is co-expressed with these two proteins in RBL-2H3 cells (Nat Calloway, unpublished results). However, the consequence of this enhanced interaction between Orai1 and STIM1 on Ca²⁺ influx has not been determined yet. Further investigation is required to test which of the above mechanisms is involved in the regulation of store-operated Ca²⁺ influx by PI(4,5)P₂ in mast cells.

Plasma membrane targeting of PIP5kinase-I isoforms and the spatio-temporal regulation of PI(4,5)P₂ synthesis

Previous studies have suggested non-redundancy in the biological roles of the different PIP5kinase isoforms. Our results provide evidence for the spatial segregation of two functionally distinct pools of PI(4,5)P₂ produced by PIP5kinase-Iα and -Iγ at the plasma membrane in mast cells. This derives from our observation of the Inp51-mediated effect on calcium release from stores and calcium influx, given its localization at the plasma membrane. The plasma membrane localization of transiently expressed PIP5kinase-Iγ, and of PIP5kinase-Iα in the stable clones, confirms this hypothesis. We also probed the potential localization of these PIP5kinase-I isoforms to lipid rafts by selectively targeting Inp54p to these domains. Significantly, we recapitulated the negative regulation on Ca²⁺ influx by altering the pool of PI(4,5)P₂ specifically associated with lipid rafts. This result has several consequences. First, it predicts that the pool of PI(4,5)P₂ synthesized by PIP5kinase-Iα is localized to rafts, and therefore that the kinase
isoform itself may be associated with rafts. Second, it suggests that the Ca\(^{2+}\) influx machinery or at least part of it, is associated with a raft environment. Recent studies are consistent with this possibility [20].

Prior evidence and proposed mechanisms for the localized synthesis of PI(4,5)P\(_2\) in lipid rafts is discussed in some detail in Chapter 1. Most of the data reviewed comes from in vitro studies and experiments involving depletion of cholesterol or incubation of cells at low temperatures [22, 12]. Thus far, the most convincing evidence for a pool of PI(4,5)P\(_2\) associated with rafts comes from the study of trafficking in polarized MDCK (kidney epithelial) cells. Guerriero et al. demonstrated that ectopically expressed PIP5kinase-I\(\alpha\) selectively regulates the trafficking of lipid-raft associated proteins to the raft-rich apical surface of polarized MDCK cells [10]. The transport of non-raft proteins or those destined to the basolateral surface remains unaffected in these cells. In addition, PIP5kinase-I\(\alpha\) also localizes to the apical surface in polarized cells [38]. Since rafts are enriched at the apical surface of these polarized cells [28], these results support our hypothesis for the raft-localization of PIP5kinase-I\(\alpha\). There is some suggestion that PIP5kinase-I\(\gamma\) may colocalize to basolateral membranes in polarized cells and hence, be segregated from PIP5kinase-I\(\alpha\), however, this data is not conclusive [3, 38].

2.6 Summary and Proposed Mechanisms

In summary, we show that PIP5kinase-I\(\alpha\) and -I\(\gamma\) regulate functionally distinct pools of PI(4,5)P\(_2\) at the plasma membrane in mast cells. We hypothesize that PIP5kinase-I\(\gamma\) positively regulates Ca\(^{2+}\) release from stores by providing the
substrate for PLCγ. We also propose that PIP5kinase-Iα potentially modulates the activity of TRPM4 channels or TRPC5/1 to negatively regulate Ca^{2+} influx. Finally, we show that Ca^{2+} response is negatively regulated by the pool of PI(4,5)P_2 localized in rafts, and hence, suggest that this may be the site for the recruitment of PIP5kinase-Iα. In this regard, we speculate that PIP5kinase-Iγ may be excluded from rafts, thereby allowing the two pools of PI(4,5)P_2 to be functionally and spatially segregated at the plasma membrane. Interaction of the two PIP5kinase-I isoforms with proteins that target them to these plasma membrane microdomains may be involved in this process.


CHAPTER 3
NOVEL ROLES FOR A POLYBASIC SEQUENCE IN Fc\(\epsilon\)RI\(\gamma_{cyt}\) IN THE REGULATION OF SURFACE EXPRESSION AND PHOSPHORYLATION OF THE IGE-RECEPTOR

3.1 Abstract

The surface expression and phosphorylation of the high affinity receptor for IgE, Fc\(\epsilon\)RI, are necessary events in the initiation of mast cell signaling. In this study, we show that PI(4,5)P\(_2\) positively regulates receptor phosphorylation in RBL-2H3 cells: overexpression of wt PIP5kinase-\(I\alpha\) increases stimulated phosphorylation of Fc\(\epsilon\)RI, whereas, expression of mutant PIP5kinase-\(I\alpha\) or the PI(4,5)P\(_2\)-specific 5’-phosphatase, Inp51, reduce this phosphorylation. We also show novel roles for a polybasic sequence of residues in the cytoplasmic segment of Fc\(\epsilon\)RI (Fc\(\epsilon\)RI\(\gamma_{cyt}\)), and for negatively charged phospholipids in the ER, in the surface expression of Fc\(\epsilon\)RI. We show that mutations of three or more basic residues in the polybasic sequence Fc\(\epsilon\)RI\(\gamma\) or expression of an ER-targeted MARCKS effector domain cause the retention of chimeric \(\alpha\gamma\gamma\) receptors in the ER. Finally, we hypothesize that the polybasic sequence of Fc\(\epsilon\)RI\(\gamma_{cyt}\) plays important roles in receptor export from the ER, receptor stabilization at the cell surface, and in the regulation of receptor phosphorylation, by interacting with PI(4,5)P\(_2\). Mechanisms of these effects are currently under investigation.
3.2 Introduction

The expression of FcεRI on the surface of mast cells is essential for initiation of allergic responses; mice lacking FcεRI are resistant to cutaneous and systemic anaphylaxis [7]. In rodents, FcεRI is expressed as a tetramer composed of three non-covalently linked subunits: an IgE-binding alpha subunit, a signal amplifying beta subunit, and a signal transducing, disulfide-linked gamma subunit dimer (αβγ2) [20]. In humans, FcεRI is expressed as a tetramer on mast cells and basophils, and as a trimer lacking the beta subunit (αγ2) on certain antigen presenting cells such as the Langerhans cell. Many factors that regulate the assembly, surface expression and activation of FcεRI have now been described. For instance, it is known that the tetrameric versions of FcεRI show higher levels of surface expression as well as more sustained activation compared to the trimeric versions of the receptor [23]. Recent data also suggests that polymorphisms in the FcεRIβ subunit may determine the susceptibility of individuals to allergens. Receptors containing an FcεRIβ subunit variant (FcεRIβvar) that lacks the ITAM motif show reduced capacity for receptor activation [9]. Most importantly, individuals suffering from allergic disorders display increased serum levels of IgE, as well as increased surface expression of FcεRI receptors on mast cells, suggesting a correlation between receptor expression and strength of the response to an allergic stimulus [20, 38].

Figure 3.1 shows a schematic representation of the biosynthetic pathway for FcεRI. FcεRI subunits are produced and assembled cotranslationally in the ER. The FcεRIα subunit forms the core of the receptor. βγ complexes cannot assemble in the absence of the α subunit [9]. A stretch of polybasic and hydrophobic residues in the cytoplasmic domain of FcεRIγ (aa 1-9, RLKIQVRK, see Figure
Figure 3.1: Schematic representation of the biosynthetic pathway for FcεRI. A simplified view of steps involved in receptor trafficking to the cell surface and crosslinking-triggered internalization are shown along with participating cellular organelles.
3.13), proximal to the transmembrane domain (juxtamembrane region), is required for receptor assembly; deletions in this region reduce the association of the α and γ subunits [22]. Following core glycosylation, glucose trimming, and folding in the ER [1, 9], receptor complexes are transported by COPII coated vesicles to the Golgi where they undergo further steps of glycosylation. The receptor complex undergoes strict quality control in the ER and the Golgi, and in the event of improper assembly is transported back to the ER by COPI coated vesicles [6]. Surface expression is also reduced if glycosylation of FcεRIα is inhibited [1]. The β and γ subunits promote modification and proper folding of the alpha subunit for binding IgE but are not essential for this process [9]. Finally, correctly assembled receptors are dispatched to the plasma membrane where they are further stabilized by binding to IgE [38].

Typically, ER-derived multisubunit proteins contain several retention signals that prevent the export of incorrectly assembled proteins from the ER, and retrieval motifs that allow recovery of defective proteins that have escaped to other cellular compartments. These retention/retrieval motifs are either cleaved off, neutralized by an anti-signal, or sterically masked when receptor subunits are assembled. The most commonly present ER retrieval motif consists of two lysine residues, usually at the -3 and -4 positions from the carboxy terminus of cytoplasmic domains of the type I transmembrane proteins [22]. Such di-lysine motifs are recognized by subunits of the COPII machinery in the retrograde transport of improperly assembled receptors to the ER. Two di-lysine motifs in the cytoplasmic domain of FcεRIα (FcεRIα_cyt) and an aspartate in the transmembrane domain of FcεRIα regulate the surface expression of the α subunit [6, 22]. One of the di-lysines is located at -3/-7 positions from the FcεRIα_cyt C-terminus and functions in ER retrieval, albeit less efficiently than conventional
-3/-4 di-lysine motifs [22]. The first 20 amino acids of the juxtamembrane region of FcεRIγ cyt are sufficient for masking the di-lysine in the FcεRIα cyt domain [22]. Only the extracellular domain of human FcεRIα lacks such motifs [3], and when expressed without the transmembrane or cytoplasmic segments, can be secreted efficiently from the ER. The extracellular domain of rodent FcεRIα, as well as the transmembrane and cytoplasmic domains of FcεRIα from both species require coexpression of FcεRIγ for this process [16].

The functional consequences of FcεRI crosslinking and activation have been described in chapters 1 and 2 of this thesis. The α and γ subunits of FcεRI are the minimum domains required to elicit functional responses similar to that seen following FcεRI aggregation [28]. Nevertheless, the surface expression and the signaling capability of the receptors is enhanced when the β subunit is present [23]. Once phosphorylated, the FcεRIβ subunit ITAM functions as a binding site for Lyn kinase, causing increased activation of the receptor. Upon binding, Lyn transphosphorylates tyrosine residues of the ITAM motifs in FcεRIβ and FcεRIγ of adjacent receptors, allowing recruitment and activation of downstream targets [32]. The actin cytoskeleton functions as a negative regulator of receptor activation: inhibition of actin polymerization by cytochalasin D enhances the amplitude and duration of stimulated tyrosine phosphorylation of the receptor subunits [11, 19]. These events require the crosslinking-dependent recruitment of receptor aggregates to ordered microdomains (lipid rafts) in the plasma membrane [18]. The transmembrane sequences of the receptor subunits determine the ability of the receptor to partition into these domains [15]. Lyn kinase in these microdomains is protected from inactivating phosphatases [40], and hence, has higher specific activity than Lyn present outside of these microdomains [39]. Hence, the lipid microenvironment, FcεRIβ subunit, and the
actin cytoskeleton are three crucial regulators of receptor activation.

Recent studies suggest that lipids at the plasma membrane may, in fact, actively interact with and regulate surface receptors. For instance, binding of the cytoplasmic domains of EGF receptors to plasma membrane PI(4,5)P$_2$ isolates the kinase domains, and maintains the receptors in an inactive conformation [30]. This effect is reversed upon agonist stimulated release of calcium-calmodulin that competes off the PI(4,5)P$_2$ and allows the kinase domains to juxtapose and transphosphorylate. Recently, Sigalov et. al [31] proposed that the Fc$\epsilon$RI$\gamma$ subunit interacts with acidic phospholipids in vitro.

In light of this growing evidence for the participation of membrane lipids in receptor regulation, we evaluated the effect of PI(4,5)P$_2$ levels on early steps of IgE-receptor signaling. We found that stimulated receptor tyrosine phosphorylation was positively regulated by PI(4,5)P$_2$. To systematically investigate potential sites for the interaction of PI(4,5)P$_2$ with Fc$\epsilon$RI, we constructed chimeric $\alpha\gamma\gamma$ receptors containing the extracellular domain of human Fc$\epsilon$RI$\alpha$ fused to the transmembrane and cytoplasmic domains of Fc$\epsilon$RI$\gamma$. Specifically, we investigated putative-PI(4,5)P$_2$ binding basic residues in the juxtamembrane region of Fc$\epsilon$RI$\gamma_{cyt}$. Surprisingly, we found that mutations of 3 out of the 4 basic residues in this region to alanine completely abolished the surface expression of mutant $\alpha\gamma\gamma$ receptors in CHO cells. Further, we could block the surface expression of wt $\alpha\gamma\gamma$ receptors by the expression of an ER targeted MARCKS effector domain. Our results suggest that the polybasic sequence in the juxtamembrane region of Fc$\epsilon$RI$\gamma_{cyt}$ participates in receptor surface expression. Our results also suggest a novel role for PI(4,5)P$_2$ in the positive regulation of stimulated receptor tyrosine phosphorylation and internalization in mast cells.
3.3 Materials and methods

3.3.1 Materials

Monoclonal anti-2,4-dinitrophenyl (DNP) IgE was purified as previously described [27]. Alexa488 anti-DNP mouse IgE, Alexa488 anti-DNP human IgE, and FITC-anti-DNP mouse IgE have been previously described [15, 8]. Anti-human FcεRIα subunit antibody was from Upstate Biotech (Lake Placid, NY). FcεRIα and γ cDNA were gifts from Dr. Henry Metzger. YFP-MARCKS effector domain was a gift from Dr. Tobias Meyer (Stanford U.). DNA oligos were purchased from Integrated DNA technologies (Coralville, IA). Restriction enzymes were from Invitrogen (XhoI, HindII, EcoRI, BamHI, DpnI) and New England Biolabs (BsrGI, NotI). IαWT, IαMut and Inp cells are described in Chapter 2.

3.3.2 Cell culture

RBL cell culture is previously described (Chapter 2). Chinese Hamster Ovary (CHO) cells were maintained as a monolayer in F12 nutrient medium (Invitrogen) supplemented with 10% fetal bovine serum (Atlanta Biologicals, GA), and geneticin (Invitrogen).

3.3.3 Transfection and Immunocytochemistry

For transfection, cells were plated overnight at a subconfluent density (0.5 X 10^6/mL) in 35 mm MatTek dishes (MatTek, Ashland, MA). The next day,
cells were washed into OptiMEM (Invitrogen) and incubated with DNA-lipofectamine 2000 complexes for 6-24 hours. Cells were labeled with fluorescent IgE prior to fixation with 3.7% formaldehyde.

### 3.3.4 Cloning of αγγ

A three step PCR reaction was used to generate the αγγ sequence\(^1\). The primers used are listed in Figure 3.2.

**STEP 1**: The reaction mix was prepared by mixing 82.2\(\mu\)l of distilled water, 10\(\mu\)l of 10X reaction buffer, 0.8\(\mu\)l of dNTP, 1 \(\mu\)l of 188ng/\(\mu\)l FcεRIα cDNA, 2.5\(\mu\)l of ChimIgEr5S, 2.5\(\mu\)l of ChimIgEr4AS, and 1\(\mu\)l of PfuTurbo. The cycling parameters for the PCR were 1 cycle at 95\(^0\)C for 1 min, 15 cycles at 95\(^0\)C for 1 min, 55\(^0\)C for 1 min, 72\(^0\)C for 1 min, and 1 cycle at 72\(^0\)C for 10 min. The PCR fragment was purified using the QIAquick Gel Extraction Kit (QIAGEN Inc., CA).

**STEP 2**: The reaction mix was prepared by mixing 81\(\mu\)l of distilled water, 10\(\mu\)l of 10X reaction buffer, 1\(\mu\)l of dNTPs, 1\(\mu\)l of 140ng/ml FcεRIγ cDNA, 2.5\(\mu\)l of ChimIgEr1S, 3.5\(\mu\)l of DNA from step 1, and 1\(\mu\)l of PfuTurbo (Stratagene, La Jolla, CA). The cycling parameters for the PCR were 1 cycle at 95\(^0\)C for 1 min, 20 cycles at 95\(^0\)C for 1 min, 61\(^0\)C for 1 min, 72\(^0\)C for 1.5min, and 1 cycle at 72\(^0\)C for 10 min.

**STEP 3**: The reaction mix was prepared by mixing 73\(\mu\)l of distilled water, 10\(\mu\)l of 10X reaction buffer, 1\(\mu\)l of dNTPs, 10\(\mu\)l of DNA from step 2, 2.5\(\mu\)l of ChimIgEr1S, 2.5\(\mu\)l of ChimIgEr4AS, and 1\(\mu\)l of PfuTurbo. The cycling parameters for the PCR were 1 cycle at 95\(^0\)C for 1 min, 15 cycles at 95\(^0\)C for 1 min, 55\(^0\)C for 1 min, 72\(^0\)C for 1 min, and 1 cycle at 72\(^0\)C for 10 min.

\(^1\)In collaboration with Dr. Alice Wagenknecht-Wiesner
<table>
<thead>
<tr>
<th>PCR REACTION</th>
<th>PRIMER NAME</th>
<th>SEQUENCE</th>
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<tbody>
<tr>
<td>Cloning of αγγ into pEGFP-N1</td>
<td>ChimIgEr5S</td>
<td>5’-CCG CGT GAG AAG TAC TGG CTA GTG ATC TTG TTC TTG CTC C-3’</td>
</tr>
<tr>
<td></td>
<td>ChimIgEr4AS</td>
<td>5’-CGG CCG AAG CTT CAG GCC CGT GTA GAC AGC-3’</td>
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<tr>
<td></td>
<td>ChimIgEr1S</td>
<td>5’-GCC GGC CCT CGA GGA TGG CTC CTG CCA TGG AAT CCC-3’</td>
</tr>
<tr>
<td>Cloning of αγγ into pCDNA3.1</td>
<td>aggSBamHI</td>
<td>5’-GCC GGC CGG ATC CGA TGG CTC CTG CCA TGG AAT CC-3’</td>
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<td></td>
<td>aggASEcoRI</td>
<td>5’-CCG CCG GAA TTC TTA CAG GCC CGT GTA GAC AGC-3’</td>
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Figure 3.2: List of primers used for the cloning of αγγ. αγγ cDNA was initially cloned into the pEGFP-N1 vector to produce αγγ-EGFP. A non-GFP version was created later by digesting the αγγ insert and ligating into pCDNA3.1 vector.
2. Cloning of $\alpha\gamma\gamma$ into pEGFP-N1 (BD Biosciences, NJ): The DNA from the third step of the PCR reaction and the pEGFP vector were digested using XhoI and HindIII restriction enzymes, and ligated using the Rapid DNA Ligation kit (Roche Applied Science). The ligated DNA was transformed using DH5$\alpha$ cells (Invitrogen, Carlsbad, CA), and selected on ampicillin plates.

3. Cloning of $\alpha\gamma\gamma$ into pCDNA3.1 (Invitrogen, Carlsbad, CA): EcoRI and BamHI restriction sites were introduced by PCR. The reaction mix was prepared by mixing 82.5$\mu$L of distilled water, 10$\mu$L of 10X reaction buffer, 1$\mu$L of dNTPs, 0.5$\mu$L of $\alpha\gamma\gamma$-EGFP, 2.5$\mu$L of aggsBamHI, 2.5$\mu$L of aggASEcoRI, and 1$\mu$L of PfuTurbo. The cycling parameters for the PCR were 1 cycle at 95$^\circ$C for 2 min, 20 cycles at 95$^\circ$C for 30 sec, 55$^\circ$C for 1 min, 72$^\circ$C for 1 min, and 1 cycle at 72$^\circ$C for 10 min. The insert was ligated into the pCDNA3.1 vector using the rapid DNA Ligation kit, transformed using DH5$\alpha$ cells and selected on ampicillin plates.

3.3.5 Mutagenesis of transmembrane sequence of $\alpha\gamma\gamma$

Wt $\alpha\gamma\gamma$ was subject to point mutations$^2$ using the quick change site-directed mutagenesis kit (Stratagene, La Jolla, CA). Primers used are listed in Figure 3.3.

The reaction mix was prepared by mixing 5$\mu$L of 10X reaction buffer, 1$\mu$L of 50ng/$\mu$L template, 1.25$\mu$L of sense primer, 1.25$\mu$L of antisense primer, 1$\mu$L of dNTP mix, 1$\mu$L of PfuTurbo (2.5U/$\mu$L) and 39.5$\mu$L of distilled water. The cycling parameters for the PCR were 1 cycle at 95$^\circ$C for 30 sec, 16 cycles at 95$^\circ$C for 30 sec, 55$^\circ$C for 1 min, and 68$^\circ$C for 6 min. The PCR product was digested for 1 hr at 37$^\circ$C using 1$\mu$L (10U/$\mu$L) Dpn1 restriction enzyme and transformed into

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$^2$In collaboration with Dr. Alice Wagenknecht-Wiesner
<table>
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<tr>
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<th>MUTATION</th>
<th>PRIMER NAME</th>
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<tr>
<td>αγγ mut 1</td>
<td>RKLQVAK</td>
<td>JMmut1S</td>
<td>5'-CGA CTC AAG ATC CAG GTC GCA AAG GCA GAC ATA GCC AGC-3’</td>
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<td></td>
<td></td>
<td>JMmut1AS</td>
<td>5'-GCT GGC TAT GTC TGC CTT TGC GAC CTG GAT CTT GAG TGC-3’</td>
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<tr>
<td>αγγ mut 2</td>
<td>RKLQVAA</td>
<td>JMmut2S</td>
<td>5'-CGA CTC AAG ATC CAG GTC GCA GCG GCA GAC ATA GCC GCG C-3’</td>
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<td>JMmut2AS</td>
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<tr>
<td>αγγ mut 3</td>
<td>ALKIQVAA</td>
<td>JMmut3S</td>
<td>5'-CCT TAC CCT GCT CTA CTG ACT CAA GAT CCA GGT CGC-3’</td>
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<td></td>
<td>JMmut3AS</td>
<td>5'-GCC ACC TGG ATC TTG AGT GCA CAG TAG AGC AGG GTA AGG-3’</td>
</tr>
<tr>
<td>αγγ mut 4</td>
<td>ALAIQVAA</td>
<td>JMmut4S</td>
<td>5'-CCT GCT CTA CTG TGC ACT CGC GAT CCA GGT CGC AGC GGC-3’</td>
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<tr>
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<td>JMmut4AS</td>
<td>5'-GCC GCT GCG ACC TGG ATC GCG AGT GCA CAG TAG AGC AGG-3’</td>
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</table>

Figure 3.3: List of primers used for mutating polybasic residues in the juxtamembrane region of αγγ. The above primers were used in the site directed mutagenesis of αγγ. Basic amino acids chosen for mutation are in bold and mutated residues are in red.
XL1-Blue supercompetent cells (Stratagene, La Jolla, CA). DNA was extracted using QIAGEN maxiprep kit (QIAGEN Inc., Valencia, CA).

3.3.6 Cloning of ER-targeted constructs

Primers used are listed in Figure 3.5.

Cloning of Cal-EGFP-MARCKS-KDEL:

**STEP1:** Cloning the calreticulin signal sequence into the pEGFP-N1 vector (BD Biosciences, NJ). pEGFP vector was digested using BglII and EcoRI restriction enzymes. CalsigS and CalsigAS oligos were annealed by mixing and heating to 95°C for 2 min and cooling slowly to RT. The annealed fragment was then ligated into the digested vector using the Rapid DNA Ligation Kit.

**STEP 2:** Cloning the MARCKS-KDEL sequence into the vector obtained in **STEP 1**. The reaction mix was prepared by mixing 40.6µL distilled water, 5µL 10X buffer, 0.4µL dNTPs, 1µL YFP-MARCKS-ED, 1µL MarBsrGS, 1µL MarNotAS, and 1µL PfuTurbo. The parameters for the PCR were 1 cycle at 95°C for 2 min, 30 cycles at 95°C for 30 min, 55°C for 30 min and 72°C for 1 min, and 1 cycle at 72°C for 10 min.

The vector from **STEP 1** and PCR product from **STEP 2** were digested individually using the BsrGI and NotI restriction sites. The digested products were annealed using the Rapid DNA Ligation kit.

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3In collaboration with Dr. Alice Wagenknecht-Wiesner
Figure 3.4: **ER-targeted MARCKS effector domain and control EGFP construct used in this study.** A mammalian expression vector encoding the enhanced green fluorescent protein (EGFP) and the endoplasmic reticulum (ER) targeting sequence of calreticulin fused to the 5’ end of EGFP was used. The effector domain of the MARCKS peptide and the ER retention sequence, KDEL, were fused to the 3’ end of EGFP. The MARCKS effector domain was excluded to generate the control EGFP construct.
<table>
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<th>PCR REACTION</th>
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<th>SEQUENCE</th>
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<tr>
<td>Cloning of Cal-EGFP-MARCKS ED-KDEL</td>
<td>CalsigS</td>
<td>5’-GAT CTA ATG CTG CTA TCC GTG CCG TTG CTG CTC GGC CTC CTC GGC CTG GCC GTC GCC GG-3’</td>
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<td>CalsigAS</td>
<td>5’-AAT TCC GGC GAC GGC CAG GCC GAG GCC GAG CAG CAA CGG CAC GGA TAG CAG CAT TA-3’</td>
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<tr>
<td></td>
<td>MarBsrGS</td>
<td>5’-GGC GGT GTA CAA GGA ATT CAA AAA AAA GAA GCG C-3’</td>
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<tr>
<td></td>
<td>MarNotAS</td>
<td>5’-GCG GCG CGG CCG CTT ACA GCT CGT CCT TCT TGT ACG ATC TCT TCT TCT TGA AGG AG-3’</td>
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<tr>
<td>Cloning of Cal-EGFP-KDEL</td>
<td>KDELoligoS</td>
<td>5’-GTA CAA GCT CGA GCT CAA GCT TAG ATC GTA CAA GAA GGA CGA GCT GTA AGC-3’</td>
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<td>KDELoligoAS</td>
<td>5’-GGC CGC TTA CAG CTC GTC CTT GCT GTA CGA TCT AAG CTT GAG CTC GAG CTT-3’</td>
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Figure 3.5: **Primers used for cloning the ER-targeted MARCKS constructs.** The above primers were used for ligating the MARCKS effector domain into the pEGFP-cal vector, and for the addition of the ER retention (KDEL) sequence as described in Figure 3.4.
Cloning of Cal-pEGFP-KDEL:

**STEP 1: Cloning the calreticulin signal sequence into the pEGFP-N1 vector.** The pEGFP-N1 vector was digested using BglII and EcoRI restriction enzymes. CalsigS and CalsigAS oligos were annealed by mixing and heating to 95°C for 2 min and cooling slowly to RT. The annealed oligos were ligated into the digested vector using the Rapid DNA Ligation Kit.

**STEP 2: Cloning the KDEL sequence into the vector obtained in STEP1.** The vector from STEP 1 was digested using the BsrGI and NotI restriction enzymes. KDELoligoS and KDELoligoAS were annealed by mixing and heating to 95°C for 2 min and cooling slowly to RT. The annealed oligos were ligated into the digested vector using the Rapid DNA Ligation Kit.

**3.3.7 Tyrosine phosphorylation**

Cells stably expressing PIP5kinase-Iα or Inp51 were sensitized overnight with anti-DNP IgE and stimulated in suspension with 50ng/mL DNP-BSA for 1, 3 or 5 min at 37°C. The receptor was immunoprecipitated using mouse anti-IgE and protein A beads (Amersham, Piscataway, NJ) for 2 hrs. Blots were detected using anti-phosphotyrosine antibody (4G10, Upstate Biotechnology, Lake Placid, NY), biotin-SP goat anti-mouse IgG (Jackson immunoresearch, West Grove, PA) and HRP conjugated anti-goat NeutrAvidin (Pierce, Rockford, IL).
3.3.8 Receptor endocytosis

Cells were resuspended in medium supplemented with 20mM HEPES at a concentration of $4 \times 10^6$ /mL, and labeled with $3 \mu$g/mL FITC-IgE for 2 hrs at $37^\circ$C. Cells were washed and resuspended in tyrodes buffer containing 1mg/mL BSA. Final concentration of cells in the cuvette was $2 \times 10^6$ /mL. Quenching of FITC-IgE was measured in a SLM 8100C steady state fluorimeter (SLM instruments, Urbana, IL) as previously described [37].

3.4 Results

3.4.1 PI(4,5)P$_2$ positively regulates stimulated tyrosine phosphorylation of Fc$\epsilon$RI

To investigate a potential role for PI(4,5)P$_2$ in receptor activation, we looked at the tyrosine phosphorylation of Fc$\epsilon$RI subunits in RBL-2H3 cells and stable variants I$\alpha$WT, I$\alpha$Mut and Inp cells described in Chapter 2. Cells were stimulated in suspension with 50ng/mL antigen for 0, 1, 3 or 10 min, and tyrosine phosphorylation of immunoprecipitated receptor subunits was detected in western blots using an anti-phosphotyrosine antibody (4G10) as described in Materials and Methods. As shown in Figure 3.6, I$\alpha$WT cells showed enhanced tyrosine phosphorylation of the $\beta$ and $\gamma$ subunits of Fc$\epsilon$RI at 1, 3 and 10 min after antigen stimulation. In contrast, I$\alpha$Mut cells showed a corresponding reduction in tyrosine phosphorylation levels compared to 2H3 cells (Figure 3.7), suggesting that PI(4,5)P$_2$ levels positively modulate stimulated receptor phosphorylation.
Inp cells also showed a decrease in receptor phosphorylation compared to 2H3 cells, with maximal reduction in phosphorylation levels detected at 1 and 10 min after stimulation (Figure 3.8). The observed reduction in phosphorylation levels of Inp cells was not due to reduced recovery of FcεRI subunits (Figure 3.8, lower panel, β blot).

3.4.2 Depletion of PI(4,5)P₂ at the plasma membrane accelerates actin-dependent internalization of FcεRI.

Receptor activation is terminated by internalization of receptors and their delivery to lysosomes [12]. To determine the effect of PI(4,5)P₂ on this process, we examined the stimulation-dependent internalization of FITC-IgE bound to FcεRI in Inp cells. Quenching of FITC fluorescence in the acidic environment of the endosomes was used as an indicator of internalization in a fluorimeter-based assay. Quenching profiles representative of three experiments are shown in Figure 3.9 for 2H3 and Inp cells. Internalization was observed beginning at approx. 5 min after crosslinking with anti-IgE and was monitored for a total of 30 min. Quenching of FITC fluorescence integrated over the time of the response showed on average, a 2.5 fold increase in Inp cells compared to 2H3 cells (Figure 3.10), suggesting enhanced receptor internalization in the absence of PI(4,5)P₂ at the plasma membrane in these cells. No quenching of FITC fluorescence was detected in the absence of stimulation or in the presence of cytochalasin D (Figure 3.10) as stimulated internalization of FcεRI is blocked by this inhibitor of actin polymerization [37].
Figure 3.6: **Wt PIP5kinase-1** expression enhances FcεRI phosphorylation. Cells were sensitized with anti-DNP IgE and stimulated with 50 ng/mL DNP-BSA (+Ag) for the time indicated. Wt clone (WT) was compared with untransfected RBL-2H3 cells. Receptor was immunoprecipitated using mouse anti-IgE and detected using anti-phosphotyrosine antibody (4G10). Data shown is representative of two experiments.
Figure 3.7: Mutant PIP5kinase-Iα expression inhibits FcεRI phosphorylation. Cells were sensitized with anti-DNP IgE and stimulated with DNP-BSA (+Ag) as in Figure 3.6. Mutant clone (Mut) was compared with untransfected RBL-2H3 cells. Receptor phosphorylation was detected as in Figure 3.6. Data shown is representative of two experiments.
Figure 3.8: Expression of ECFP-Inp51 reduces FcεRI phosphorylation. Cells were sensitized with anti-DNP IgE and stimulated with DNP-BSA (+Ag) as in Figure 3.6. ECFP-Inp51 clone (Inp) was compared with untransfected RBL-2H3 cells. Receptor phosphorylation was detected as in Figure 3.6. Data shown is representative of two experiments.
Figure 3.9: **Stimulated endocytosis of IgE-FcεRI is enhanced in Inp cells compared to 2H3 cells.** Receptor endocytosis was detected in a fluorimeter-based assay, using FITC-IgE to label the receptors. Plots show representative quenching of FITC fluorescence indicative of receptor endocytosis from one experiment. Arrow indicates addition of anti-IgE antiserum (1:100 dilution).
Figure 3.10: **Inp cells show a 2.5 fold increase in receptor endocytosis levels compared to 2H3 cells.** Plots show average integrated quenching of FITC fluorescence from three independent experiments for Inp cells relative to 2H3 cells. Error bars show SEM.
3.4.3 Expression of $\alpha\gamma\gamma$ constructs

A recent study implicated a sequence of polybasic residues in the regulation of EGF receptor phosphorylation by binding to PI(4,5)P$_2$ [30]. An analogous sequence was found in the juxtamembrane region of Fc$\varepsilon$RI$\gamma_{cyt}$ (Stuart McLaughlin, personal communication). To systematically investigate the role of this putative PI(4,5)P$_2$-binding juxtamembrane sequence in the phosphorylation of Fc$\varepsilon$RI, we constructed a chimeric receptor with the extracellular domain of human Fc$\varepsilon$RI$\alpha$, fused to the transmembrane and cytoplasmic domains of Fc$\varepsilon$RI$\gamma$ subunits. EGFP at the C-terminus of this protein was used to visualize expression and localization.

Our initial attempts to transiently express wt $\alpha\gamma\gamma$-EGFP failed because of surprisingly poor expression at the plasma membrane in RBL-2H3 cells (data not shown). An EGFP tagged chimeric construct comprising of the extracellular domain of human Fc$\varepsilon$RI$\alpha$, fused to the transmembrane domain of Tac and cytoplasmic domains of TCR$\zeta$, used as a control, showed good plasma membrane expression in these cells ($\alpha\mathrm{T\zeta}$-EGFP, Figure 3.11). We tested if an untagged version of the chimeric receptor showed better expression [28]. Wt $\alpha\gamma\gamma$ was transiently expressed in RBL-2H3 cells using lipofectamine, and surface expression of receptors was probed by labeling with Alexa488-anti-DNP huIgE for 2 hrs at 37°C, 24 hours post-transfection. Under these conditions, we detected expression of wt $\alpha\gamma\gamma$ at the plasma membrane and in internalized pools (Figure 3.11). The transfection efficiency of wt $\alpha\gamma\gamma$ was limiting in RBL-2H3 cells, and attempts to use electroporation to improve transfection resulted in mostly internalized receptors. Labeling for 30 min instead of 2 hrs to minimize internalization in the event that IgE was aggregated only marginally improved
surface expression. Attempts to stably express wt $\alpha\gamma\gamma$ in RBL-2H3 cells to overcome limitations of transfection efficiency also failed due to the lack of surface expression of the receptors.

To overcome the above limitations with transfection capability, we employed Chinese Hamster Ovary (CHO) cells to express wt $\alpha\gamma\gamma$. EGFP-tagged $\alpha\gamma\gamma$ did not show much surface expression even in CHO cells (Figure 3.12). For untagged $\alpha\gamma\gamma$, close to 80% of the cells showed expression. Due to the lack of endogenous Fc$\epsilon$RI in this cell line, we labeled the receptors with Alexa488-anti-DNP mouseIgE\(^4\) 24 hours post transfection for 30 min in suspension at $37^\circ$C (Figure 3.12). Receptors showed strong expression at the plasma membrane as detected by IgE. No significant internalization of receptors was observed.

Stimulated tyrosine phosphorylation in cells expressing $\alpha\gamma\gamma$ was not detected by western blotting (data not shown) despite very good expression seen by microscopy. This could be limiting because of two reasons: first, $\alpha\gamma\gamma$ phosphorylation is limited by the absence of an amplifying $\beta$ subunit [23, 15] and second, the Src family kinase present in CHO cells mediates only limited phosphorylation of Fc$\epsilon$RI ($\alpha\beta\gamma_2$) [40].

To explore the role of the polybasic residues, we created four mutants of $\alpha\gamma\gamma$ by successively modifying two arginine and two lysine residues in the juxtamembrane region of Fc$\epsilon$RI$\gamma_{cyt}$, implicated in sequestering PI(4,5)P\(_2\) (see Figure 3.13). We compared the surface expression of wt and mutant $\alpha\gamma\gamma$ constructs in CHO cells. Cells were labeled with Alexa488-mouse IgE in suspension (Figure 3.14) or when attached (Figure 3.15) for 30 min at $37^\circ$C. Cells were then fixed, permeabilized with 0.1% triton-X, and labeled with an antibody targeting

\(^4\)Mouse-IgE can bind to both human and rodent Fc$\epsilon$R\(_\alpha\) [16]
Figure 3.11: Expression of wt αγγ in RBL-2H3 mast cells. Cells transiently transfected with αγγ were labeled for 2 hours with Alexa488-anti-DNP huIgE prior to fixation. Surface expression of receptors was compared under similar conditions to wt αTζ-EGFP. Images were taken using a Leica SP2 TCS confocal microscope and scale bars show 20µm.
Figure 3.12: Expression of wt αγγ in CHO cells. Cells transiently transfected with αγγ were labeled for 30 min with Alexa488-anti-DNP mouse IgE prior to fixation. Images were taken using a Leica SP2 TCS confocal microscope and scale bars show 20µm.
the human FcRRI alpha subunit (anti-FcRRIα). Cells expressing the vector alone (Control, Figure 3.15) showed no binding of Alexa488-IgE, and showed non-specific low background staining when labeled with the anti-FcRRIα antibody. Wt αγγ showed expression at the plasma membrane. Mutant 1 and mutant 2 showed receptor expression on the surface as detected by Alexa488-IgE (Figure 3.14). However, these also showed increased presence of receptors inside the cell, compared to wt αγγ as detected by the anti-FcRRIα antibody. Mutant 3 and mutant 4 failed to show any significant surface expression with IgE. This was not due to lack of expression as non-surface pools of the receptor could be detected by the anti-FcRRIα antibody. The internal pool of mutant 3 and mutant 4 appeared to be largely confined to the ER. These results indicate that polybasic residues in the juxtamembrane region are important for the surface expression of αγγ, and loss of three or more of these residues results in ER retention.

3.4.4 Expression of an ER targeted MARCKS effector domain blocks the export of wt αγγ receptors from the ER

In RBL-2H3 cells, the ER-targeted MARCKS effector domain (EGFP-MARCKS-KDEL) localizes throughout the ER but is concentrated in punctae near the golgi complex. Co-transfection of this construct with H-Ras, which is prenylated in the ER and normally transported to the plasma membrane via the Golgi complex [14], inhibits the surface expression of this GTPase (D. Holowka, unpublished observations). The effector domain of MARCKS contains polybasic residues that can bind negatively charged phospholipids including PI4P, PI(4,5)P2 and PI(3,4,5)P3 [25, 17]. The above result suggests that negatively
Figure 3.13: **Wt and mutant αγγ constructs used in this study.** The juxtamembrane sequence (JM) is underlined. Basic amino acids are in bold. Mutated residues are in red.
Figure 3.14: Expression of wt and mutant αγγ constructs in CHO cells in suspension. CHO cells expressing wt and mutant αγγ were labeled with Alexa488-anti-DNP mouse IgE for 30 min at 37°C in suspension. Cells were fixed, permeabilized with 0.1% TritonX-100 and labeled with anti-human FceRIα subunit antibody, followed by Alexa546 anti-rabbit IgG. Human IgG (1:100) was used to block non-specific binding of the secondary antibody. Images were taken using a Leica SP2 TCS confocal microscope and scale bars show 10 µm.
Figure 3.15: **Expression of wt and mutant $\alpha\gamma\gamma$ constructs in attached CHO cells.** CHO cells expressing wt and mutant $\alpha\gamma\gamma$ were labeled with Alexa488-anti-DNP mouseIgE for 30 min at 37°C in suspension. Cells were fixed, permeabilized with 0.1% TritonX-100 and labeled with anti-human FcεRI $\alpha$ subunit antibody, followed by Alexa546 anti-rabbit IgG. Human IgG (1:100) was used to block non-specific binding of the secondary antibody. Images were taken using a Leica SP2 TCS confocal microscope and scale bars show 25 $\mu$m.
charged phospholipids mediate surface transport of ER-derived proteins via the Golgi. To probe whether interaction of the polybasic residues of $\alpha\gamma\gamma$ with negatively charged lipids might be important in mediating the export of the receptors from the ER, we transiently expressed EGFP-MARCKS-KDEL together with wt $\alpha\gamma\gamma$. Co-expression of EGFP-MARCKS-KDEL in CHO cells completely abolished surface expression of wt $\alpha\gamma\gamma$ receptors and of $\alpha\gamma\gamma$ mutant 2 (Figure 3.16, mutant 1 was not tested). The receptors colocalized with MARCKS, suggesting that interaction of $\alpha\gamma\gamma$ with a negatively charged phospholipid may be involved in its export from the ER.

Figure 3.17 compares the localization of wt $\alpha\gamma\gamma$ in the presence of EGFP-MARCKS-KDEL or EYFP-MARCKS, which localizes to the plasma membrane and the nucleus. As described before, in the absence of MARCKS, wt $\alpha\gamma\gamma$ receptors localized strongly to the plasma membrane, and this was blocked by the co-expression of EGFP-MARCKS-KDEL. We could detect wt $\alpha\gamma\gamma$ receptors at the surface when co-expressed with EYFP-MARCKS, and it was often accompanied by a large concentration of receptors in internal pools reminiscent of recycling endosomes, suggesting that interaction of $\alpha\gamma\gamma$ with acidic phospholipids, including PI(4,5)P$_2$, at the plasma membrane may be involved in its stabilization at that location.

3.5 Discussion

Antigen-stimulated tyrosine phosphorylation of Fc$\epsilon$RI subunits is the first biochemically detectable step in mast cell activation. Our results suggest a novel role for the polyphosphatidylinositol, PI(4,5)P$_2$, in the regulation of Fc$\epsilon$RI
Figure 3.16: Coexpression of ER-localized MARCKS in CHO cells blocks the export of wt αγγ receptors. MARCKS effector domain was visualized using EGFP. Wt or mutant 2 αγγ receptors were labeled in fixed cells, permeabilized with 0.1% TritonX-100, using anti-human FceRIα subunit antibody, followed by Alexa546 anti-rabbit IgG. Human IgG (1:100) was used to block non-specific binding of the secondary antibody. Images were taken using a Leica SP2 TCS confocal microscope and scale bars show 10 µm.
Figure 3.17: Effect of the coexpression of ER-localized or plasma membrane localized MARCKS on the surface expression of wt $\alpha\gamma\gamma$ receptors. MARCKS effector domain was visualized using EGFP or EYFP fluorescence as indicated. Wt $\alpha\gamma\gamma$ constructs were labeled in fixed cells, permeabilized with 0.1% TritonX-100, using anti-human Fc$\epsilon$RI$\alpha$ subunit antibody, followed by Alexa546 anti-rabbit IgG. Human IgG (1:100) was used to block non-specific binding of the secondary antibody. Images were taken using a Leica SP2 TCS confocal microscope and scale bars show 10 $\mu$m.
phosphorylation. Upregulation of PI(4,5)P$_2$ levels by the overexpression of PIP5kinase-I$_\alpha$ enhances stimulated tyrosine phosphorylation of Fc$\epsilon$RI subunits in RBL-2H3 cells, whereas overexpression of a catalytically inactive mutant PIP5kinase-I$_\alpha$ or a PI(4,5)P$_2$-specific phosphatase decreases stimulated receptor phosphorylation levels. In addition, PI(4,5)P$_2$ levels at the plasma membrane appear to correlate with the stability of crosslinked receptors at the cell surface. Depletion of PI(4,5)P$_2$ at the plasma membrane by the expression of the PI(4,5)P$_2$-specific phosphatase enhances the internalization of the receptors.

We have also identified a putative-PI(4,5)P$_2$ binding sequence in the juxtamembrane region of Fc$\epsilon$RI$_\gamma_{cyt}$ that regulates surface expression of the receptor. Mutations of three or more basic residues in this sequence is sufficient to trap chimeric $\alpha\gamma\gamma$ receptors in the ER. Surface expression of wt $\alpha\gamma\gamma$ receptors can also be inhibited by expressing an ER-targeted MARCKS effector domain. The above observations suggest that the interaction of PI(4,5)P$_2$ with the polybasic sequence in Fc$\epsilon$RI$_\gamma_{cyt}$ may be involved in the regulation of Fc$\epsilon$RI surface expression, phosphorylation and endocytosis. Potential mechanisms and supporting data from the literature are discussed below.

**Role of PI(4,5)P$_2$ and polybasic sequences in the regulation of receptor phosphorylation:** We hypothesize that binding of the juxtamembrane region of Fc$\epsilon$RI$_\gamma$ to PI(4,5)P$_2$ at the plasma membrane promotes interactions that render the Fc$\epsilon$RI$_\gamma$ ITAM sequences more favorable to phosphorylation by Lyn kinase. Sigalov et al. [31] used structural prediction algorithms and circular dichroism spectra to show that cytoplasmic domains of multichain immune recognition receptors including Fc$\epsilon$RI$_\gamma$, are mostly disordered. This random coil nature of Fc$\epsilon$RI$_\gamma_{cyt}$ did not change upon receptor oligomerization or peptide binding to acidic large unilamellar vesicles (LUVs). The net positive charge of Fc$\epsilon$RI$_\gamma_{cyt}$
(+3) was proposed to be involved in its binding to acidic phospholipids in the LUVs. In addition, phosphorylation of the ITAM sequence reduced the net charge and partially weakened the binding of the FcεRIγ_cyt peptide to the LUVs, suggesting that other mechanisms potentially involving specific basic clusters similar to what we have proposed, plays a role in the interaction of FcεRIγ_cyt with the plasma membrane in vivo.

We observe a similar charge dependence in dictating the localization of wt and mutant αγγ receptors at the plasma membrane. Reducing the net positive charge of FcεRIγ_cyt by mutating the basic residues also reduced surface expression of αγγ. More specifically, mutant 1 (+2) and mutant 2 (+1) show more internal pools of the receptor, and surface expression of αγγ is completely lost for mutant 3 and mutant 4 where the charge is reduced to 0 and -1, respectively. The interaction is also reduced when EYFP-MARCKS that localizes to the plasma membrane and sequesters the PI(4,5)P_2 at that location, is coexpressed with wt αγγ. This suggests that availability of negatively charged phospholipids at the plasma membrane and a net positive charge >0 of FcεRIγ_cyt are critical requirements for the surface expression αγγ.

Recently, polybasic residues in the juxtamembrane region in the cytoplasmic domains of the EGF receptor were shown to be part of a flexible linker that binds PI(4,5)P_2 at the plasma membrane [30]. This interaction was hypothesized to maintain the EGF receptor in an inactive conformation by segregating the kinase domains of monomeric receptors. In response to agonist, calcium calmodulin was proposed to compete off the juxtamembrane region, causing the cytoplasmic domains to de-associate from the plasma membrane and juxtapose as a result. Hence, PI(4,5)P_2 was hypothesized to serve as a reversible inhibitor of
EGFR activation. However, unlike EGF receptors where the activation depends on the proximity of the two cytoplasmic domains, phosphorylation of FcεRI involves interaction of individual subunits with Lyn kinase [18]. We hypothesize that binding of FcεRIγcyt to the plasma membrane PI(4,5)P₂ may, in fact, remove potential steric hinderance due to adjacent cytoplasmic domains, making the ITAM sequences more accessible to Lyn and activating phosphorylation.

In a study using bone marrow derived mast cells (BMMCs) from PIP5kinase-Iα knockout mice, Sasaki et al. showed that the association of FcεRI with lipid rafts was enhanced in the KO cells [29]. Since FcεRI association with lipid rafts correlates with receptor activation [10], results with PIP5kinase-Iα KO BMMCs suggest that PI(4,5)P₂ should downregulate receptor phosphorylation in contrast to our observations in RBL-2H3 cells. As Sasaki et al. do not report effects of PIP5kinase-Iα KO on stimulated tyrosine phosphorylation of receptor subunits, it is unclear whether PIP5kinase-Iα−/− BMMCs exhibit different trends in this regard than RBL-2H3 cells expressing mutant PIP5kinase-Iα. In this study, we have not investigated the functional effects of other PIP5kinase-I isoforms in regulating receptor phosphorylation. However, given the distinct functional effects of PIP5kinase-Iα and -Iγ on the calcium response in mast cells (see Chapter 2), we predict that PIP5kinase-Iγ does not affect receptor phosphorylation. This hypothesis is bolstered by our results suggesting that PIP5kinase-Iα and -Iγ are localized in a spatially segregated manner at the plasma membrane.

**Role of PI(4,5)P₂ and polybasic sequences in the stability of receptors at the plasma membrane:** We observe an increase in internal pools of αγγ when PI(4,5)P₂ is masked by MARCKS at the plasma membrane and an increase in stimulated endocytosis of FcεRI in cells expressing the PI(4,5)P₂-specific phos-
phatase, Inp51. FcεRI is stabilized at the plasma membrane by binding to IgE [38]. We suggest that binding of FcεRIγcyt to PI(4,5)P₂ serves as an additional mechanism to stabilize the receptor at the plasma membrane. This may decrease internalization and enhance sustained phosphorylation of receptor subunits by Lyn kinase. The nonspecific electrostatic interaction between FcεRIγcyt and PI(4,5)P₂ may also be potentially involved in masking the -4/-7 pseudodilysine motif, that may otherwise increase endocytosis.

**Role of polybasic residues in the regulation of receptor export from the ER:** We hypothesize that COPII subunits directly recognize the polybasic residues of FcεRIγcyt for packaging receptors into vesicles destined for the golgi. The juxtamembrane region is ideally positioned on the cytoplasmic side of the ER for such cargo-recognition to occur. In this model, a negatively charged phospholipid, most probably PI4P or PI(4,5)P₂, is involved in the independent recruitment of COPII subunits. This could explain why either mutating the polybasic residues or potentially blocking coat recruitment by expression of ER-targeted MARCKS can block receptor export from the ER.

Unlike the plasma membrane where polyphosphoinositides are enriched in the cytoplasmic leaflet, the membrane of the ER is symmetrical with respect to distribution of these phospholipids [21]. This is despite the asymmetric synthesis of these phospholipids on the cytoplasmic face of the ER membrane. A yet to be identified protein, named flippase, is proposed to redistribute lipids between the cytoplasmic and luminal leaflets of the ER membrane. In fact, Vishwakarma et al. demonstrated that PI could be efficiently “flipped” across the ER membrane [34]. In view of this data, we can explain how EGFP-MARCKS-KDEL that is retained in the ER lumen can still bind to negatively charged phospholipids.
synthesized on the cytoplasmic face of the ER membrane. However, whether flippase activity towards the lumen is enhanced in response to MARCKS expression in the ER is not clear. If true, this could be a potential mechanism for depleting the negatively charged phospholipid pool on the cytoplasmic side of the ER to prevent the export of wt αγγ.

COPII coated vesicles transport cargo from the ER to the Golgi complex. The mammalian COPII coat complex consists of the Sar1-GTPase, Sec23\Sec24, and Sec13\Sec31 proteins \[2, 35\]. Acidic phospholipids, especially PI4P and PI(4,5)P₂, are suggested to function in coat recruitment and vesicle budding from the ER. In yeast, Wiradjaja et al. \[36\] demonstrated that null mutants of an ER-targeted PI(4,5)P₂-specific phosphatase, Inp54, showed at least a 2-3 fold increase in secretion, suggesting that PI(4,5)P₂ positively regulates this process. Matsuoka et al. \[24\] demonstrated that in vitro, PI4P and PI(4,5)P₂ are required for the binding of COPII coat proteins to liposomess.

In addition, phosphoinositides play a role in defining sites for vesicle budding. One classical example is Vps34 (Vacuolar protein sorting), a yeast PI3-kinase. Mutants of Vps34 fail to sort hydrolases to vacuoles \[33\]. It was later demonstrated that localized production of PI3P by Vps34 in the trans golgi network defines sites for the generation of vesicles targeted specifically to vacuoles \[5\]. Similarly, overexpression of a FAPP-PH domain, capable of binding PI4P, inhibits the assembly of COPII subunits in vitro, and vesicle budding at ER exit sites \[4\]. This suggests that analogous to the role for PI3P in the golgi, PI4P may define sites for the budding of COPII coated vesicles from the ER.

To date, four mammalian homologs of Sec24 are known \[35, 26\]. It is suggested that different homologs bind different types of cargo. In addition, Sec24
is proposed to contain multiple independent sites for cargo recognition. Generally, di-acidic or di-hydrophobic sequences on type I transmembrane proteins direct their selection into COPII-coated vesicles [2]. Recently, di-basic residues have also been reported to function as ER exit signals in type II transmembrane proteins [13, 35]. These di-basic residues are more N-terminal compared to the dilysines involved in ER retrieval which are typically at -3 and -4 positions from the carboxy terminus of proteins. For instance, an RK(X)RK sequence in the proteins of the Golgi glycosyltransferases family [13] is recognized by Sar1. Alanine substitution of these basic residues blocks ER exit of the glycosyltransferases. In addition, the export of an ER resident protein, Iip33, can be triggered by substitution of the cytoplasmic tail of Iip33 with that of a glycosyltransferase. Given the above, it is tempting to speculate that a specific isoform of Sec24 is involved in packaging of polybasic ER exit sequences, like the one suggested by this study in FcεRIγ_cyt, for export from the ER.

3.6 Proposed model

Our study has identified a novel candidate sequence in the regulation of surface expression and stimulated tyrosine phosphorylation of the IgE receptor, FcεRI. We implicate a juxtamembrane sequence of polybasic residues in FcεRIγ_cyt in these processes, and we propose a model wherein this sequence initially enables sorting of correctly assembled receptor subunits to COPII coated vesicles destined to the golgi. The recruitment of the coat subunits may be dependent on PI4P or PI(4,5)P_2 in the ER. Once FcεRI reaches the plasma membrane, PI(4,5)P_2 may help stabilize receptor expression at the cell surface via non specific electrostatic interactions with the same polybasic residues in the juxtamembrane
region. This could, in addition, render the ITAM sequence of FcεRIγcyt more accessible to phosphorylation by Lyn kinase or for binding downstream targets like Syk. Agonist stimulated depletion of PI(4,5)P₂ could destabilize the receptors and induce internalization to lysosomes. In summary, our results suggest previously uncharacterized roles for two new candidates, FcεRIγcyt polybasic sequence, and PI(4,5)P₂, in the regulation of FcεRI expression and function in mast cells.


[29] SASAKI, J., SASAKI, T., YAMAZAKI, M., MATSUOKA, K., TAYA, C., SHITARA, H., TAKASUGA, S., NISHIO, M., MIZUNO, K., WADA, T., MIYAZAKI, H., WATANABE, H., IIZUKA, R., KUBO, S., MURATA, S.,


[37] XU, K., WILLIAMS, R. M., HOLOWKA, D., AND BAIRD, B. Stimulated release of fluorescently labeled IgE fragments that efficiently accumulate


CHAPTER 4
SUMMARY AND FUTURE DIRECTIONS

Science is always wrong.
It never solves a problem without creating ten more.
– George Bernard Shaw

The capacity of mast cells to express and phosphorylate FccRI, and to mobilize Ca^{2+} determines their capacity to degranulate in response to allergic and inflammatory triggers [3, 5]. This dissertation describes two investigations that identify novel roles for the membrane glycerophospholipid, PI(4,5)P_{2}, in mast cells. We used overexpression and murine knockout of PI(4,5)P_{2}-metabolizing enzymes as tools to alter the levels of this phospholipid in mast cells. We investigated two PI(4,5)P_{2}-synthesizing enzymes (PIP5kinase-I_α and -I_γ), two PI(4,5)P_{2}-depleting phosphatases (Inp51 and Inp54), and a PI(4,5)P_{2} sequestering MARCKS effector domain in this process. In addition, we also employed chimeric αγγ receptors to explore interactions between PI(4,5)P_{2} and FccRI_γ_{cyt}. Our results indicate roles for PI(4,5)P_{2} in regulating the cell surface expression and phosphorylation of FccRI (Chapter 3), and in the dual regulation of calcium mobilization (Chapter 2) in mast cells. In addition, our data supports the existence of at least two functionally distinct pools of PI(4,5)P_{2} at the plasma membrane of these cells. Below we summarize major results and propose an integrated model for the biological role of PI(4,5)P_{2} in mast cells.
4.1 The big picture: an integrated model for the role of PI(4,5)P$_2$ in mast cells

The subunits of Fc$\varepsilon$RI ($\alpha\beta\gamma_2\gamma_2\gamma$) are assembled in the endoplasmic reticulum in mast cells [4]. Thereafter, the receptor is trafficked by COP-coated vesicles to the Golgi complex, where it is glycosylated prior to transport to the plasma membrane. Once at the plasma membrane, Fc$\varepsilon$RI binds to circulating IgE and is stabilized at the cell surface [16]. We find that expression of an ER-targeted MARCKS effector domain blocks the export of chimeric wt $\alpha\gamma\gamma$ receptors, suggesting that a negatively charged phospholipid (possibly PI4P or PI(4,5)P$_2$) in the ER membrane is involved in the recruitment of COPII subunits to sites of vesicle budding in this organelle [7]. We also observe that mutations of three or more basic residues that are part of a polybasic sequence in the juxtamembrane region of Fc$\varepsilon$RI$\gamma_{cyt}$ abolish cell surface expression of $\alpha\gamma\gamma$ receptors in CHO cells. We hypothesize that this polybasic sequence functions as an ER exit signal and be recognized by the COPII subunits to allow the receptor complex to be transported to the Golgi for further modification [1, 9].

We observe increased internalization of receptors in cells when we reduce available PI(4,5)P$_2$ pools at the plasma membrane by expressing Inp51 or MARCKS effector domain, suggesting that binding of the polybasic sequence of Fc$\varepsilon$RI$_{\gamma_{cyt}}$ to PI(4,5)P$_2$ on the inner leaflet of the plasma membrane contributes to receptor stability at the cell surface. In addition, we observed an enhancement of stimulated phosphorylation of Fc$\varepsilon$RI in cells expressing wt PIP5kinase-I$\alpha$. We propose that binding of the polybasic sequence of Fc$\varepsilon$RI$\gamma$ to PI(4,5)P$_2$ favorably exposes the ITAM tyrosine to Lyn kinase upon the antigen-mediated clustering...
Figure 4.1: **Summary of important results:** (1) Polybasic sequence in FcεRIγcyt and negatively charged phospholipids in the ER regulate export of chimeric αγγ receptors from the ER. (2) Binding of PI(4,5)P2 to the polybasic sequence in FcεRIγcyt may regulate receptor stability at the plasma membrane. (3) PI(4,5)P2 negatively regulates internalization of FcεRI. (4) PI(4,5)P2 positively regulates phosphorylation of FcεRI. (5) PIP5kinase-Iγ synthesizes the PLC-γ-hydrolyzable pool of PI(4,5)P2 and positively regulates Ca^{2+} release from the ER. (6) PIP5kinase-Iα synthesizes a pool of PI(4,5)P2 that negatively regulates Ca^{2+} influx.
of FcεRI in lipid rafts [13].

Activation of FcεRI triggers phosphorylation of downstream signaling intermediates that include PLCγ [12]. We observe a reduction of antigen-stimulated Ca^{2+} release from the ER in BMMCs from PIP5kinase-Iγ^{−/−} mice, and in RBL-2H3 cells expressing the plasma membrane localized Inp51. This suggests that PLCγ hydrolyzes a spatially distinct pool of PI(4,5)P₂ synthesized by PIP5kinase-Iγ at the plasma membrane to generate IP₃. Depletion of ER Ca^{2+} stores triggers store-operated calcium entry [10], and we observe a reduction in Ca^{2+} influx stimulated by antigen or thapsigargin in cells overexpressing wt PIP5kinase-Iα, suggesting that PI(4,5)P₂ thus synthesized negatively regulates the Ca^{2+} influx machinery, possibly by interacting with TRPM4 or TRPC5 channels [6, 15]. In addition, targeting Inp54 specifically to lipid rafts relieves the inhibition on Ca^{2+} influx by PI(4,5)P₂, suggesting that this pool of PI(4,5)P₂ synthesized by PIP5kinase-Iα associates with ordered membrane domains.

These results provide evidence for previously uncharacterized roles for PI(4,5)P₂ and other polyphosphatidylinositols in FcεRI biosynthesis and trafficking, as well as in FcεRI signaling leading to Ca^{2+} mobilization. In addition, they reveal functionally distinct pools of PI(4,5)P₂ at the plasma membrane that are separately synthesized by the two different PIP5kinase-I isoforms.

### 4.2 Future directions

The mechanisms and potential candidates proposed above are currently under investigation. Below, we report work in progress, future directions, and preliminary solutions to some of the technical challenges raised during our study.
Mechanism for localized PI(4,5)P$_2$ production.

Much of the above proposed model relies on the spatial segregation of PI(4,5)P$_2$ pools produced by PIP5kinase-I$\alpha$ and -I$\gamma$ at the plasma membrane. Although the distinct functional effects of the kinase isoforms on Ca$^{2+}$ mobilization strongly support this view, we need to understand the structural basis for this functional segregation by more direct means. One approach is to investigate the localization of PIP5kinase isoforms to isolated raft or non-raft fractions by western blotting. One of our greatest limitations has been the unavailability of isoform specific antibodies that detect PIP5kinase-I$\alpha$ and -I$\gamma$ reliably by blotting or immunocytochemistry (see Appendix A). However, we have access to epitope tagged versions of the kinase isoforms that we can investigate with respect to their localization to raft domains at the plasma membrane.

Another approach to investigate whether PIP5kinase-I$\gamma$ also localizes to rafts is to use flow cytometry to evaluate whether calcium release from stores is affected upon expression of raft localized Inp54 (L10-Inp54-GFP). The raft and non-raft localized Inp54 constructs offer a simple way to identify more functionally distinct pools of PI(4,5)P$_2$. However, we have been limited by the unexpected displacement of the non-raft (Src-tagged) construct to the cytoplasm, and by the technical difficulties in detecting the Ca$^{2+}$ response in a small subset of transfected RBL cells. The Src targeting sequence contains polybasic residues (in addition to myristoylation) that support recruitment to the plasma membrane [11]. The depletion of PI(4,5)P$_2$ by Inp54 explains the mislocalization of the S15-Inp54-GFP construct to the cytoplasm. However, the observed increased cytoplasmic localization of the control S15-GFP compared to L10-GFP suggests that the Src tag is not as effective for plasma membrane targeting. One solu-
tion might be to employ a different targeting sequence such as that from Ras to target the phosphatase to non-raft regions [8].

**Effect of PIP5kinase-IG isozymes on IP₃ levels.**

We see a reduction in the antigen-mediated calcium release from stores in PIP5kinase-I°C−/− BMMCs. Our model postulates that this isoform regulates the PLCγ-mediated production of IP₃ levels. Measurement of IP₃ levels in wt and PIP5kinase-I°C−/− BMMCs would allow us to test this hypothesis more definitively. This experiment has been limited thus far by insufficient numbers of PIP5kinase-I°C−/− BMMCs. An interesting point to note is that the average calcium release from stores is reduced only by 50% over multiple experiments, although we see some indication of complete loss of stimulated Ca²⁺ release from stores at lower antigen doses. These data at lower antigen doses needs to be explored further. In addition, the measurement of IP₃ levels will tell us if there are other sources of PI(4,5)P₂ for IP₃ production, or if a potential IP₃-independent mechanism (e.g., via sphingosine kinase) can account for residual Ca²⁺ release from ER stores in PIP5kinase-I°C−/− BMMCs.

We made some attempts to determine the splice variant of PIP5kinase-IG that is specifically involved in the regulation of calcium release from stores (see Appendix A). We transiently overexpressed wt and mutant PIP5kinase-IG87 and -IG90 in RBL-2H3 cells and monitored their total calcium response by flow cytometry but failed to see significant differences in several experiments. In this case, monitoring just the antigen mediated release from stores by excluding extracellular calcium, and using a lower antigen dose may help resolve potential differences better.
Identification of the influx channel being modulated by PI(4,5)P₂ in the negative regulation of calcium influx.

In chapter 2, we propose three possible targets for the action of PI(4,5)P₂ on Ca²⁺ influx, namely TRPM4, TRPC5 and TRPC1. The TRPC channels are already under investigation in our laboratory, and it appears that shRNA mediated knockdown of TRPC1 has a strong effect on the antigen-mediated initiation of the calcium response of RBL-2H3 cells (Roy Cohen, unpublished observations) whereas the knockdown of TRPC5 more strongly affects thapsigargin-stimulated influx [6]. From the literature, we know that PI(4,5)P₂ influences multiple TRPC channels and activates TRPM4 channels [14, 15]. We therefore, predict that the negative regulatory effect of PI(4,5)P₂ on calcium influx involves effects on multiple calcium influx channels.

Mechanisms of regulation of FcεRI surface expression and phosphorylation.

There are two primary issues: first, the mechanism by which the polybasic sequence governs the exit of αγγ (and FcεRI) receptors from the ER, and second, the mechanism by which MARCKS blocks the exit of wt αγγ receptors from the ER. In the discussion for chapter 3, we allude to some potential mechanisms. We propose that the polybasic sequence of FcεRIγcyt is recognized by Sec24, and hence when we mutate the basic residues in this sequence, we lose the ER export capacity. To test this, we can insert an independent Sec24 recognition sequence (DXE motif) in αγγ mutant 4 (that did not show cell surface expression) and ask if this motif can rescue the ER export of the mutant¹. Mutations of the polybasic residues could interfere with the folding of the chimeric receptor. However,

¹Suggestion by Prof. W. J. Brown (Cornell U.)
due to the unstructured nature of FcεRIγ cytoplasmic segment [13], we think that improper folding does not play a role in restricting ER export of receptors.

With respect to the role of the MARCKS peptide, we first need to investigate whether the inhibition of ER export is specific to proteins that traffic through the Golgi, or if this is a bulk effect. Experiments to test this are already in progress where we are comparing the effects of the co-expression of EGFP-MARCKS-KDEL with other plasma membrane targeted proteins such as Lyn, Protein tyrosine phosphatase alpha, and H-Ras. Other possible solutions may involve in vitro systems to look at the rate of vesicle budding from the ER, similar to those used by Perry et al. [2].

If we are able to rescue the surface expression of the mutant $\alpha\gamma\gamma$ by the use of the independent Sec24 binding motif as suggested above, we may be able to compare the effect of the polybasic sequence on the phosphorylation of the different wt and mutant $\alpha\gamma\gamma$ constructs. However, there are limitations to this experiment. If PI(4,5)P$_2$ at the plasma membrane is essential for the receptors to be stable at the cell surface, this approach may not work. In addition, we have thus far been unable to detect sufficient phosphorylation of $\alpha\gamma\gamma$ in CHO cells by western blotting. To overcome this challenge, we may either have to carry out this experiment in RBL-2H3 cells where transfection is limited, or co-express Lyn with $\alpha\gamma\gamma$ in Cos7 cells [17]. If we can determine the conditions to reduce the internalization of $\alpha\gamma\gamma$ receptors in RBL cells, limitations of transfection efficiency may be overcome by using flow cytometry for detecting phosphorylation signals in individual cells. Thus far, our observations using the $\alpha\gamma\gamma$ chimeric IgE receptor and the ER-targeted MARCKS construct have revealed unexpected indications for roles of negatively charged phospholipids in the ER
to plasma membrane protein trafficking, and in plasma membrane stabilization of these proteins. Future studies should help to understand the generality and mechanisms for these effects.
BIBLIOGRAPHY


APPENDIX A
ADDITIONAL RESULTS FROM PIP5KINASE-I STUDY

A.1 Distribution of endogenous PIP5kinase-I\(\alpha\) and \(\gamma\) by immunocytochemistry

We explored the distribution of endogenous PIP5kinase-I\(\alpha\) and -I\(\gamma\) in RBL-2H3 cells by immunocytochemistry and western blotting. Figures A.1 and A.3 show endogenous distributions of PIP5kinase-I\(\alpha\) and -I\(\gamma\), respectively, representative of two independent experiments each. Cells were plated at \(0.5 \times 10^6\)/mL in 35mm MatTek wells overnight and fixed using 3.7% formaldehyde for 15 min on the next day. For immunocytochemistry, cells were permeabilized with either 0.1% TritonX-100 or 1mg/mL Saponin as indicated. A custom made antibody (PAC2025, Pacific Immunology, Ramona, CA), raised against a C-terminal sequence (ILSSISQEWKDEKQDL) was used to detect PIP5kinase-I\(\alpha\) in Figure A.1. Protein disulphide isomerase (PDI) was used as an ER marker. In resting cells permeabilized with saponin, this anti-PIP5kinase-I\(\alpha\) labels the plasma membrane weakly. A large fraction of the endogenous PIP5kinase-I\(\alpha\) is detected in the cytosol and appears to partially co-localize with PDI. The label is also concentrated in a perinuclear region that may be Golgi associated. Co-localization of this perinuclear pool with Golgi marker GM-130 was detected in one instance (data not shown). Cells permeabilized with tritonX-100 show a more uniform distribution of PIP5kinase-I\(\alpha\) that is also predominantly cytosolic and does not colocalize well with the PDI. We observe some localization of PIP5kinase-I\(\alpha\) at the plasma membrane in cells that are more spread out and ruffling (Figure A.1; lower left panel). A commercial antibody (goat polyclonal anti-PIP5kinase-
Iα, sc-11775, Santa Cruz Biotechnology Inc., CA, [10]) labels a predominantly plasma membrane associated pool of PIP5kinase-Iα (Figure A.2). Some cytosolic structures are also detected by this antibody.

The above observations are in agreement with reports in the literature for PIP5kinase-Iα localization in overexpression studies. PIP5kinase-Iα localizes primarily to punctate cytosolic structures in addition to some marginal localization to the plasma membrane [3, 5, 8, 12]. This is also consistent with our observations with ectopically expressed wt PIP5kinase-Iα (Chapter 2; Figure 2.2) in RBL-2H3 cells where we see localization of the kinase in the cytosol and at the plasma membrane. Selective activation and increased membrane recruitment of PIP5kinase-Iα is observed in HeLa cells following hyperosmotic stress [12]. PIP5kinase-Iα also colocalizes with Arf6 in membrane ruffles [5].

PIP5kinase-Iγ was detected using the γPAN antibody\textsuperscript{1}, that detects all splice variants [11]. In resting cells permeabilized with saponin, PIP5kinase-Iγ localizes weakly to the plasma membrane and to distinct punctate structures in the cytoplasm that are almost completely excluded from the ER (Figure A.3). The plasma membrane pool becomes slightly more evident in antigen stimulated cells (data not shown) although the labeling is very faint to be imaged. We also observe a very brightly labeled nuclear pool of PIP5kinase-Iγ. In cells permeabilized with tritonX-100, most of the label is nuclear and the plasma membrane pool is not detected.

Previous reports in the literature suggest that most of PIP5kinase-Iγ is strongly associated with the plasma membrane, with the γ87 isoform distributed more uniformly and the γ90 isoform recruited specifically to focal ad-

\textsuperscript{1}The γPAN antibody was a generous gift from P. De Camilli, Yale U.
Figure A.1: Endogenous PIP5kinase-I\(\alpha\) is detected in the cytoplasm and the plasma membrane. PIP5kinase-I\(\alpha\) was labeled in fixed RBL-2H3 cells permeabilized either with 0.1% TritonX-100 or 1mg/mL Saponin, using PAC2025 rabbit polyclonal antibody followed by Alexa488 anti-rabbit IgG. Human IgG (1:100) was used to block non-specific binding of the secondary antibody. PDI was labeled using anti-PDI mAb followed by Alexa555 anti-mouse IgG\(_{2b}\) antibody. Images were taken using a Leica SP2 TCS confocal microscope and scale bars show 20\(\mu\)m.
Figure A.2: Commercial antibody raised against PIP5kinase-Iα detects a pool that is predominantly plasma membrane localized. PIP5kinase-Iα was labeled in fixed RBL-2H3 cells permeabilized with 0.1% TritonX-100, using goat polyclonal anti-PIP5kinase-Iα followed by Alexa488 anti-goat IgG. Image was taken using a Leica SP2 TCS confocal microscope.
hesions [2, 4, 6]. The localization of the γ93 isozyme has not been explored extensively. This reported plasma membrane localization is consistent with our observation with ectopically expressed PIP5kinase-Iγ (Chapter 2; Figure 2.22).

PAC and γPAN were also used to detect PIP5kinase-Iα (64 KDa) and -Iγ (∼90 KDa) by blotting of whole cell lysates. Although these antibodies detected bands corresponding to expected molecular weights of the kinase isoforms in two initial experiments (data not shown), we were unable to detect these bands reproducibly in subsequent experiments.

A.2 Summary of other conditions on the Ca\textsuperscript{2+} response for PIP5kinase-Iα constructs.

To assess the contribution of Orai1 and IP\textsubscript{3}R at the plasma membrane to Ca\textsuperscript{2+} influx in RBL-2H3 cells, we examined the effect of CRAC-inhibitor, GdCl\textsubscript{3}, and PLCγ inhibitor, U73122, on this process. Ca\textsuperscript{2+} responses were measured using indo-1 as described in Chapter 2 (Materials and Methods). Representative plots showing the effects of these inhibitors on antigen and thapsigargin-mediated responses in RBL-2H3 cells are shown in Figure A.4 and Figure A.5, respectively.

RBL-2H3 cells show a robust Ca\textsuperscript{2+} response to stimulation by 0.2 µg/mL DNP-BSA (antigen). The large influx phase represented by the plateau of the response is inhibited by over 80\% by the addition of 6 µM GdCl\textsubscript{3}. A second addition of 6 µM GdCl\textsubscript{3} does not cause further suppression of the response. Subsequent addition of 2 µM U73122 completely inhibits stimulated Ca\textsuperscript{2+} influx.

Thapsigargin mediated Ca\textsuperscript{2+} influx is almost completely sensitive to addi-
Figure A.3: **Endogenous PIP5kinase-Iγ isoforms are at the plasma membrane, cytosol and nucleus in RBL-2H3 cells.** PIP5kinase-Iγ was labeled in fixed RBL-2H3 cells permeabilized either with 0.1% TritonX-100 or 1mg/mL Saponin, using γPAN rabbit polyclonal antibody followed by Alexa488 anti-rabbit IgG. Human IgG (1:100) was used to block non-specific binding of the secondary antibody. PDI was labeled using anti-PDI mAb followed by Alexa555 anti-mouse IgG2b antibody. Images were taken using a Leica SP2 TCS confocal microscope and scale bars show 20µm.
tion of 6µM GdCl$_3$, suggesting that almost all of the store-operated Ca$^{2+}$ influx in RBL-2H3 cells is due to CRAC.

We also monitored the effect of high potassium concentration in the extracellular buffer. We compared the Ca$^{2+}$ response of RBL-2H3 cells in regular tyrodes-BSA to cells in tyrodes-BSA with 140mM KCl. Figure A.6 shows responses representative of two independent experiments. In agreement with results published previously [7], the Ca$^{2+}$ response was diminished in the presence of 140mM KCl compared to regular tyrodes-BSA. Most of the observed response appeared to be due to Ca$^{2+}$ release from stores as the sustained plateau phase was completely lost in the presence of 140mM KCl and the response returns to baseline.

We compared the above results to conditions where the plasma membrane was hyperpolarized by the exclusion of KCl from tyrodes-BSA. Thapsigargin was used to trigger Ca$^{2+}$ release from store. Figure A.7 shows results from one experiment. The Ca$^{2+}$ response in the absence of KCl was comparable to that for cells in regular tyrodes-BSA, except for a small reduction in the plateau phase.

In summary, these results suggest that the majority of the calcium influx in RBL-2H3 cells is mediated by CRAC. The reduction in calcium influx under depolarizing conditions provides evidence for TRPM4 like calcium activated channels in the negative regulation of calcium influx in these cells [9]. The effects of the above conditions and inhibitors on the Ca$^{2+}$ response in I$_{\alpha}$WT and I$_{\alpha}$Mut cells were qualitatively similar to those shown for RBL-2H3 cells.
Figure A.4: Effect of Gd^{3+} and U73122 on antigen-stimulated store-operated Ca^{2+} influx in RBL-2H3 cells. Trace shows representative Ca^{2+} response to antigen stimulation (0.2µg/mL) in RBL-2H3 cells and effect of 6µM GdCl_{3} and 2µM U73122 from one experiment.
Figure A.5: Effect of Gd$^{3+}$ on thapsigargin-mediated store-operated Ca$^{2+}$ influx in RBL-2H3 cells. Trace shows representative Ca$^{2+}$ response to thapsigargin stimulation (0.25µM) in RBL-2H3 cells and effect of 6µM GdCl$_3$ from one experiment.
Figure A.6: Effect of high potassium concentration on antigen-stimulated Ca\textsuperscript{2+} response in RBL-2H3 cells. Trace shows representative Ca\textsuperscript{2+} response to antigen stimulation (0.2\(\mu\)g/mL) in RBL-2H3 cells and effect of 140mM KCl in the extracellular buffer from one experiment.
Figure A.7: Effect of potassium exclusion on thapsigargin-mediated Ca\textsuperscript{2+} response in RBL-2H3 cells. Trace shows representative Ca\textsuperscript{2+} response to thapsigargin stimulation (0.25\,\mu M) in RBL-2H3 cells and effect of no KCl in the extracellular buffer from one experiment.
A.3 Summary of the effect of PIP5kinase-IA on degranulation.

To investigate the effect of decreased Ca\textsuperscript{2+} mobilization in IA\textsubscript{WT} cells compared to 2H3 cells on degranulation, we looked at the percent \( \beta \)-hexosaminidase release in these cells. In brief, cells were harvested and resuspended to a concentration of \( 0.5 \times 10^6 \) /mL, and 100 \( \mu \)L was plated per well of a clear bottom 96 well plate. Cells were sensitized with IgE and allowed to incubate overnight at 37\textdegree C. The next day, cells were treated with various concentrations of DNP-BSA (Ag) as indicated for 1 hr at 37\textdegree C. 25\( \mu \)L of the supernatant was incubated with 100\( \mu \)L of substrate (4-methylumbelliferyl-N-acetyl-beta-D-glucosaminide (C18H21NO8), Sigma) for 30 min at 37\textdegree C in a dark bottom 96 well plate. The enzymatic reaction was stopped by addition of 180\( \mu \)L of glycine carbonate buffer at 4\textdegree C. The fluorescence of digested substrate was measured using a plate reader (SpectraFluor, Tefcon). Percent response was calculated by normalizing to maximal release in the presence of 0.1\% TritonX-100, and background fluorescence of tyrodes-BSA. Figure A.8 shows the average degranulation response to antigen from two independent experiments. The error bars show standard deviation between average \( \beta \)-hexosaminidase release from three wt clones (wt43, wt46 and wt49), and for two mutant clones (M31 and M37).

At all concentrations of antigen, IA\textsubscript{WT} cells showed a degranulation response that was marginally bigger than that seen in IA\textsubscript{Mut} cells and 2H3 cells. This is contrary to our expectation, and it is unclear why degranulation is not reduced under conditions where we see a reduction in the total Ca\textsuperscript{2+} release. Degranulation is dependent on intracellular Ca\textsuperscript{2+} levels and activation of PKC. Based on the results described in chapter 2, we expect that Ca\textsuperscript{2+} release from stores, and potential production of DAG by the hydrolysis of PI(4,5)P\textsubscript{2} is unaf-
fected in these cells. Hence, we expect that the activation of PKC is not likely to be affected. Therefore, despite the reduction in Ca$^{2+}$ levels, the intracellular Ca$^{2+}$ levels achieved in I\textalpha WT cells in conjunction with activated PKC are apparently sufficient to trigger strong degranulation. As PI(4,5)P$_2$ is involved in the binding of synaptotagmin and consequent granule fusion [1], its enhanced production by wt PIP5kinase-I\textalpha could facilitate enhanced degranulation in compensation for reduced Ca$^{2+}$ levels.

### A.4 Summary of degranulation and ruffling response for Inp cells.

To investigate the effect of increased Ca$^{2+}$ mobilization in Inp cells compared to 2H3 cells, we looked at the percent $\beta$-hexosaminidase release in these cells as described for PIP5kinase-I\textalpha constructs. Figure A.9 shows the dose-response relationship for the degranulation response at varying antigen concentrations. Interestingly, the degranulation response for Inp cells is reduced compared to 2H3 cells at low doses of antigen (50pg/mL and 0.5ng/mL). However, this decrease is compensated at higher doses. As a reminder, the Ca$^{2+}$ response is measured at an antigen concentration of 0.2$\mu$g/mL. At a dose of 0.5$\mu$g/mL, which is the closest to the dose of antigen used to stimulate Ca$^{2+}$ responses, we actually see a small increase in the degranulation level in Inp cells compared to 2H3 cell, corresponding to the increase in the Ca$^{2+}$ response in these cells. Hence, the degranulation response in Inp cells appears to be correlated with the calcium response in these cells.

We also investigated the effect of PI(4,5)P$_2$ depletion on stimulated ruffling
Figure A.8: Degranulation responses in IαWT and IαMut clones, and 2H3 cells. Bars represent average degranulation response to antigen from two independent experiments. Error bars show SEM.
Figure A.9: Degranulation response for Inp cells compared to 2H3 cells. Bars represent average degranulation response to antigen from two independent experiments. Error bars show SEM.
Based on the data from PIP5kinase-Iα overexpression, we expected PI(4,5)P_2 to positively regulate ruffling, and therefore for stimulated ruffling to be reduced in Inp cells. The extent of ruffling was quantified based on the percentage of cells that ruffled upon antigen stimulation (Figure A.11). Cells were scored based on their morphology as ruffled or not ruffled. In one experiment where we scored 300 cells, we did not see significant reduction in the ruffling capability of Inp cells compared to 2H3 cells. It is possible that the pool of PI(4,5)P_2 most strongly affected by Inp51 is different from the pool of PI(4,5)P_2 that contributes to the ruffling response.

A.5 Degranulation response for wt and PIP5kinase-Iγ−/− BMMCs.

To investigate the effects of reduced Ca^{2+} release from the ER in PIP5kinase-Iγ−/− BMMCs on degranulation, we measured the percent β-hexosaminidase release from wt and ko BMMCs. Cells in suspension were treated with 0.01μg/mL DNP-BSA (Ag), or 2μg/mL Ca^{2+} ionophore (A23) for 30 min at 37°C in a shaking water bath. Cells were centrifuged for 5 min at 1000 rpm. 50μL of the supernatant was incubated with 100μL of substrate (4-methylumbelliferyl-N-acetyl-beta-D-glucosaminide (C18H21NO8), Sigma) for 30 min at 37°C in a dark bottom 96 well plate. The enzymatic reaction was stopped by addition of 180μL of glycine carbonate buffer at 4°C. The fluorescence of digested substrate was measured using a plate reader. Percent value response was calculated by normalizing to maximal release in the presence of 0.1% TritonX-100, and background fluorescence of tyrodes-BSA. Figure A.12 shows the average degranu-
Figure A.10: **Stimulated ruffling in Inp cells.** Representative images for antigen-stimulated (0.8µg/mL) ruffling in RBL-2H3 and Inp cells are shown. Membrane ruffles were labeled with rhodamine-phalloidin post fixation. Images for -Ag show equatorial sections; +Ag images show dorsal surfaces. Images were taken using a Leica SP2 TCS confocal microscope and scale bars show 50µm.
Figure A.11: **Stimulated ruffling is not inhibited in Inp cells.** Bars show the percentage of cells that ruffled upon antigen stimulation from one experiment. 300 cells each for 2H3 or Inp cells were scored based on morphology as ruffled or not ruffled.
lation response to antigen from four independent experiments and to A23 from one experiment.

Surprisingly, despite the reduction in Ca\(^{2+}\) release from stores, the PIP5kinase-I\(_{\gamma}^{-/-}\) BMMCS show almost a 1.5-fold greater percent degranulation response compared to wt BMMCs in response to antigen stimulation. The degranulation response in the presence of Ca\(^{2+}\) ionophore is not significantly different for wt and ko BMMCs, suggesting that the enhancement is dependent on Fc\(_{\epsilon}\)RI activation. We tested the effect of various different inhibitors that affect intermediates downstream of Fc\(_{\epsilon}\)RI such as wortmannin (PI3-kinase), bisindolylmaleimide (BIM; Protein kinase C) and EGTA (extracellular Ca\(^{2+}\)). However, the PKC inhibitor BIM produced only a modest inhibition of the response in wt and ko BMMCs. The Lyn inhibitor, PP1, did not significantly inhibit the response in two experiments (data not shown). Apparently at the concentration used (20 \(\mu\)M), PP1 did not sufficiently inhibit Src family kinase activity to suppress the degranulation response.

In summary, PIP5kinase-I\(_{\gamma}\) plays a negative regulatory role in Fc\(_{\epsilon}\)RI signaling leading to degranulation, in addition to its positive role in stimulated Ca\(^{2+}\) mobilization. This inhibition appears to be independent of Lyn but appears to be dependent on the activation of PI3kinase and extracellular Ca\(^{2+}\) in response to antigen stimulation.
Figure A.12: **Degranulation response for wt and PIP5kinase-Iγ−/− BMMCs.** Bars represent average degranulation response to antigen from four independent experiments and to A23 from one experiment. Error bars show SEM. The inhibitors were used at the following concentrations: Wortmannin 100nM, BIM 2.2µM, and EGTA 10mM.
A.6 Summary of total Ca\(^{2+}\) response for cells overexpressing PIP5kinase-I\(\gamma\) by flow cytometry.

To determine the splice variant of PIP5kinase-I\(\gamma\) that regulated Ca\(^{2+}\) release from ER stores, we transiently overexpressed EGFP-tagged wt and mutant PIP5kinase-I\(\gamma\)87 and \(\gamma\)90 [2] in RBL-2H3 cells. The Ca\(^{2+}\) response of kinase expressing cells (GFP positive) was compared to untransfected cells (GFP negative) within the same sample using flow cytometry. The quenching of fura red fluorescence was used as an indicator of the total antigen-stimulated Ca\(^{2+}\) release. Data from two independent experiments are shown in Figure A.13. Plots show the average normalized Ca\(^{2+}\) release after antigen stimulation, at room temperature (\(23^\circ\)C). The integrated Ca\(^{2+}\) response for cells expressing wt PIP5kinase-I\(\gamma\)87 was three-fold higher than that for untransfected cells. Cells expressing mutant PIP5kinase-I\(\gamma\)90 showed a smaller (1.4-fold) increase in the overall Ca\(^{2+}\) response. Mutant PIP5kinase-I\(\gamma\)87 and PIP5kinase-I\(\gamma\)90 showed a response similar to that of untransfected cells (data not shown). However, in two subsequent experiments at 37\(^\circ\)C, we did not observe significant differences in the Ca\(^{2+}\) response of any PIP5kinase-I\(\gamma\) isozyme compared to untransfected cells (data not shown). The reason for the differences in Ca\(^{2+}\) release trends observed at the two temperatures is not clear. Even though preliminary results suggest that PIP5kinase-I\(\gamma\)87 may be the main player in regulating Ca\(^{2+}\) release from stores, more data is required before any definitive role for this isoform can be inferred.
Figure A.13: Total Ca\textsuperscript{2+} response in cells expressing wt and mutant PIP5kinase-I\textgreek{87} and -I\textgreek{90} by flow cytometry. Ca\textsuperscript{2+} responses were integrated for 9 min, and results from two independent experiments at room temperature were averaged. Bars represent responses relative to cells expressing wt PIP5kinase-I\textgreek{87}, and error bars show standard deviation.
A.7 Summary of ruffling response for cells overexpressing PIP5kinase-Iγ.

To compare the role for PIP5kinase-Iγ isoforms to PIP5kinase-Iα in actin polymerization, we looked at the effect of wt and mutant -Iγ isoforms on stimulated ruffling in 2H3 cells. Transiently transfected cells were sensitized with IgE overnight and stimulated with antigen for 15 min at 37°C. Figure A.14 shows representative images of stimulated cells, fixed and labeled with rhodamine-phalloidin to visualize the ruffles. Figure A.15 shows the percentage of cells that show stimulated ruffling. Approx. 300 cells for each condition were scored as ruffled or not ruffled, based on the morphology of cells. About 60-80% of the cells expressing either EGFP-tagged wt or mutant PIP5kinase-Iγ87 and -Iγ90 do not show significant ruffling compared to neighboring untransfected cells (Figure A.14). Ectopically expressed PIP5kinase-I has previously been shown to decrease binding of actin regulatory proteins and inhibit ruffling in CV1 cells [12]. Cells expressing an EGFP encoding vector (pIRES2-EGFP) show membrane ruffles comparable to untransfected cells, indicating that above observed suppression of ruffling is not due to EGFP expression in the cells. Since this inhibition appears to be independent of the catalytic activity of PIP5kinase-Iγ, we hypothesize that the kinase is binding to and inhibiting a regulatory protein required for actin polymerization.

2In collaboration with K. Elzer
Figure A.14: Expression of wt or mutant PIP5kinase-Iγ suppresses stimulated cell ruffling. Representative images for antigen-stimulated (0.8µg/mL) ruffling in RBL-2H3 expressing GFP-tagged wt and mutant PIP5kinase-Iγ are shown. Cells expressing GFP alone were used as control. Membrane ruffles were labeled with rhodamine-phalloidin post fixation. Images show dorsal surfaces and were taken using a Leica SP2 TCS confocal microscope. Scale bars show 15µm.
Figure A.15: Quantitation of stimulated ruffling in RBL-2H3 cells expressing wt or mutant PIP5kinase-1γ. Bars show the percentage of cells that ruffled upon antigen stimulation from three experiments, and error bars show standard deviation. About 300 cells for each condition were scored based on morphology as ruffled or not ruffled.


[9] Vennekens, R., Olausson, J., Meissner, M., Bloch, W., Mathar, I., Philipp, S. E., Schmitz, F., Weissergerber, P., Nilius, B., Flockerzi, V., and Freichel, M. Increased IgE-dependent mast cell activation and


APPENDIX B

PROTOCOLS

B.1 Protocol for extracting bone marrow derived mast cells from newborn mice legs.

Protocol adapted from [1].

B.1.1 Materials and Reagents

1. Sterile scissors
2. Sterile forceps
3. 10cc syringe
4. 30½G needle (1 per pup)
5. 20G1 needle (1 to fill syringe)
6. 60mm dishes (1 per pup)
7. 35mm dishes (1 per pup + 1 for ethanol)
8. BMMC media (900mL DMEM (Invitrogen, CA) supplemented with 10% FBS (Atlanta Biologicals) and 1mL gentamicin, prewarmed, about 12mL/pup)
9. SCF (2uL/10mL media, Peprotech Inc., NJ)
10. IL3 (2uL/10ml media, Peprotech Inc., NJ)
11. ethanol
B.1.2 Protocol

1. Keep legs on ice till you are ready to extract the cells.

2. Pour 2mL media into the 35mm dishes, and ethanol into one separate 35mm dish.

3. Fill the 10cc syringe with media containing SCF and IL3. Use a 20G1 needle to fill the syringe but change to $30\frac{1}{2}$G needle to extract the cells.

4. To extract cells, remove microfuge tube containing legs from ice and pick up one leg using sterile forceps. Close and put the tube with the remaining legs back on ice.

5. Dip the leg in ethanol briefly and place in the 35mm dish.

6. Trim both ends of the leg using sterile scissors when holding the leg using forceps. Do not place back in the 35mm dish.

7. Flush about 2.5cc media through the leg and collect in a 60mm dish.

8. Place the leg back in the 35mm dish.

9. Repeat steps 4-8 for the remaining 3 legs.

10. Remember to use new 35mm and 60mm dish, and, $30\frac{1}{2}$G needle per pup. Sterilize the forceps and scissors using ethanol when switching to a new pup.

11. At the end, pick out all the legs from the 35mm dishes. Incubate the 35mm and 60mm dish at $37^\circ$C.

12. 1-2 days after extraction, pass the cells.

(a) Pool supernatants from the 35mm and 60mm dishes for individual pups. Rinse once and collect the media.
(b) Spin at 1000rpm for 12min. Resuspend in 2-4 mL depending on the cell density and plate in a 35 or 60mm dish accordingly.

(c) Pass once every 4 days by centrifugation and resuspension in fresh medium.

B.2 Small scale viral transfection using lentivirus.

B.2.1 Safety requirements

1. Wear lab coat, double gloves, mask and eye protection when handling the viruses\(^1\).

2. After work, wash arms thoroughly and apply hand sanitizer (Purell).

3. Lab members must be warned of potential danger and advised to wear gloves in the tissue culture room.

4. Use a dedicated fridge/freezer area, and shelf in incubator.

5. Place all required material in the hood before the start of work.

6. Change gloves every time work outside the hood is required (e.g: centrifugation).

7. After work, disinfect all surfaces with 10% bleach, then wipe down with distilled water and finally with 70% ethanol. (This includes hood, all handles/doors to fridge and incubator, all bench surfaces, and equipment surfaces). Decontaminate all the portable equipment (pipettors) by wiping

\(^{1}\text{More information about lentiviral plasmids and their use can be found at http://web.mit.edu/jacks-lab/protocols/pll37.htm}\)
with 10% bleach, distilled water and 70% ethanol, and leave under UV overnight (OR at least 5 hours).

8. Dispose pipette tips and other tubes/flasks used for experiments in a small autoclave bag that is then sealed and disposed into the general biohazard waste bag.

9. Plastic pipettes must be soaked in 10% bleach solution for several days (OR at least overnight), then drained and put into an autoclave bag, sealed and disposed into the general biohazard waste bag for pipettes.

10. Liquid waste is disposed into 50 mL tubes containing full strength bleach, allowed to disinfect, and then diluted and dumped in the sink. The tubes are disposed according to #5.

11. RBL cells transfected using viral plasmid are washed thoroughly to remove virus particles in supernatant before experiment.

### B.2.2 Cell culture- 293FT cells

Protocols, 293FT cells, and all viral packaging plasmids (listed under Lentiviral Infection Protocol) were from Dr. Ryan Young (National Jewish Medical Center, Denver, CO).

293FT cells are maintained in D10 media (recipe: 1.1 L DMEM, 130 ml heat inactivated FCS (heat at 55°C for 30min), 2.12 mls each of Pen/Strep (10,000 units/mL Penicillin G and 10,000 µg/mL Streptomycin sulfate), L-glutamine (200 mM), and MEM non-essential amino acids (10mM/100X)). Viruses are harvested in D2 media (Same as D10, except 2% FBS instead of 10%). To start new cells, quick thaw frozen stock at 37°C and put contents of tubes (1ml) into 14
mls of D10 waiting in a 15 ml conical tube, Spin down at 1000 rpm for 5 min (skipping this step will decrease yield of cells), resuspend cells in 10 mls, and plate into a 100 mm dish. Cells are maintained as a monolayer at 37°C with 5% CO₂. For cell passage, harvest with trypsin for 2-3min when they become 70% confluent (in 3-4 days), pass 1:12 into 10mls of fresh D10 in new 100 mm plates. (Note 60mm plates may also be used). Note: Do not let the cells become more than 80% confluent and pass no more than 15 times as viral production will fall steeply if these precautions are not followed.

B.2.3 Lentivirus Infection Protocol - small scale

Day 1: Plate 293FT cells

Plate out 293FT cells at 10⁶ cells per 60 mm dish in 4ml of D10 medium NOTE: For next day experiment, resuspend cells from 60mm dish in 12mL of media and plate 2.5mL per 60mm dish.

Day 2: Transfection of viral plasmids

1. Check cells before transfection - should be at 80 - 90% confluency.

2. Cells may be transfected using lipofectamine or calcium phosphate. For calcium phosphate transfection, prepare the transfection mixture:

   (a) First, mix together the following in a 15 ml conical tube: NOTE: Pre-mix all DNA
TOTAL VOLUME 500µl

ddH₂O 140µl

pI (pMDL) (gag-pol) 3.3µg

pII (pREV) 1.3 µg

pIII (pVSVG) 1.9 µg

pLL3.7 5µg

1.25M CaCl₂ 100µl

2X HBS 250µl

(b) Next, bubbling air through the above transfection mixture using a 2mL disposable pipette, slowly add (drop-by-drop) 2X HBS over 30 sec-1 min. Note: The transfection mixture must be slightly milky due to a light precipitate (too heavy a precipitate will kill the cells and/or result in low transfection efficiency)

(c) You should be able to see the precipitate of calcium phosphate as a suspension of tiny dots floating around under the microscope. If there is no visible precipitate, there is a high probability your experiment will not work.

3. Add 0.5 ml of the transfection mixture drop-wise to the 293FT cells, and gently rock cells to further mix NOTE: Do not remove/change media before the experiment as the pH will affect the transfection efficiency.

4. Allow cells to incubate with the transfection mixture for overnight at 37°C with 3% CO₂ (5% is fine) NOTE: The pH of the cell media is critical to good transfection efficiency, thus watch the pH of the 2X HBS and incubate cells in 3% CO₂ for this night only.
Day 3: Wash

1. First thing in the morning, aspirate old medium from the 293FT cells and gently pipet 1mL sterile PBS. NOTE: Pipet slowly to sidewall of dish, as 293FT cells will come off.

2. Rock plate and aspirate off PBS and replace with 4.5 mls fresh D2 (DMEM with 2% FCS)

3. Incubate transfected 293FT cells at 37°C and 10% CO₂

Day 4: Infection-Round 1

1. Prepare a labeled 15ml conical tube for each sample.

2. Pipet off medium from 293 FT cells and place in 15ml conical.

3. Replace medium over cells with 4.5 ml of fresh D10, place back in incubator.

4. Add 45µl (100-fold dilution) of 1M HEPES, pH 7.4 to medium in tube.

5. Add 70µl (70ish-fold dilution) of polybrene (in D10) to medium in tube.

6. Spin tubes at 2500 rpm for 10 minutes to pellet any cell debris.

7. In the mean time, set up cells to infect:

   (a) Harvest and count the RBL cells. Aliquot 1×10⁶ cells per sample.

   (b) Resuspend each sample to be infected in 4mL of viral supernatant.

8. Place cells on rotator for 1 hr (at 37°C).

9. Spin, resuspend and plate in 5mL RBL medium overnight.
Day 5: Infection - Round 2

1. Repeat Round 1
2. Harvest cells from flask.
3. Resuspend in 4mL viral supernatant
4. Place cells on rotator for 1 hr (at 37°C).
5. Spin and resuspend in 5mL RBL medium overnight.

Day 6: Use cells for experiment.

B.2.4 Cloning shRNAs into pLL3.7:

Annealing the oligonucleotides.

1. Dilute oligonucleotides to 1µg/l.
2. Prepare annealing buffer by mixing 100mM potassium acetate (400µl of 40 µM potassium acetate), 2mM magnesium acetate (20µl 40 mM magnesium acetate), 30mM HEPES, pH 7.4 (12µl HEPES, pH 7.4) in 328µl ddH2O.
3. Mix the following together in PCR tubes: 48µl annealing buffer, 1µl sense oligo and 1µl antisense oligo
4. Anneal the oligos-this will take around 6 hours

Vector Preparation.

1. Set up digest of pLL3.7 with Hpa I and Xho I (50µl volume: 3µl pLL3.7 DNA, 39µl ddH2O, 5µl NEB Reaction Buffer 4, 2µl Hpa I, 2µl Xho I).
2. Mix well with pipetman, quick spin liquid to the bottom of the tube and place in the 37°C water bath.

3. Digest for 3 hours at 37°C.

4. Add 1 µl of CIP to digest - then mix well with pipetman followed by quick spin.

5. Incubate for an additional 30 minutes. Meanwhile prepare a 1% agarose gel.

6. Add 6 µl of 10X DNA loading buffer.

7. Run the digest on the 1% agarose gel at 120 volts for about 20 minutes.

8. Visualize the DNA band for cut pLL3.7 and excise with new razor.

9. Extract DNA from gel slice using the QIAEX II gel purification kit.

Ligation

1. Thaw buffers 1 and 2 from the Quick Ligation Kit (Roche) - then vortex each for about 10 seconds

2. Mix the following together to 15 µl total volume: 7.5µl Buffer 1, 3.0µl Buffer 2 (vortex well), 0.5µl cut pLL3.7, 3.0µl annealed oligos, 1.0µl T4 DNA ligase. Mix well with a pipetman, and then quick spin.

3. Incubate at room temp for 10 minutes.

Transformation

1. Use 20µl of NOVA BLUE competent cells.
2. Add 3µl of the above ligation mixture to competent cells and incubate on ice for 30 minutes.

3. Heat-shock by placing tubes in the 42°C water bath for 40 seconds.

4. Immediately place tubes back on ice for 2 minutes.

5. Add 200µl SOC media to each tube, and place in the 37°C bacterial incubator for 45 minutes.

6. Plate out on LB-agar plates with the appropriate selection marker (ampicillin).

7. Incubate plates overnight in the 37°C bacterial incubator.

8. Check for colonies the following morning.

**Screening the colonies for the correct plasmid.**

1. Pick colonies from the plate with a pipetman (using the pipet tip) and place in 4mls Lauria Broth with ampicillin (pick smaller colonies, they tend to have the right plasmids).

2. Grow these miniprep cultures overnight.

3. The following morning, miniprep the cultures and do a control digest as follows:

   a. Mix the following together to 20µl total volume: 10µl miniprep DNA, 7µl ddH₂O, 2µl NEB buffer 2, 0.5µl Xba I, 0.5µl Xho I (Don’t forget to digest pLL3.7 as a control).

   b. Mix well, quick-spin, and place in the 37°C water bath for 1 hour. Make a 2% agarose gel in the meantime.

   c. Add 3µl of 10X DNA loading buffer to the digest, mix and spin down.
(d) Run digests on the 2% agarose gel at 120 volts for 1 hour (or more).

(e) Visualize and document gel with geldoc, positive clones will have a 60 bp shift upward compared to the pLL3.7 control.