Host-Pathogen Interactions: Roles for the Modulation of Lipids and Actin

by

David Mason

A thesis submitted in conformity with the requirements for the degree of Doctorate of Philosophy in Biochemistry

Biochemistry Department
University of Toronto

© Copyright David Mason 2010
Abstract

Elements that are foreign to the human body, such as bacteria, viruses and fungi, are recognised by cells of the innate immune system. Through a process termed phagocytosis, microorganisms are bound, internalised and destroyed. In this thesis, we focus upon how host cells respond to IgG-opsonised targets, studying both the initial stages of Fc-receptor (FcR) ligation and the later stages of phagocytic cup formation.

We provide evidence that after clustering of the receptors, the mobility of diacylated probes such as those found in Src-family of kinases, was reduced. This immobilisation was found to be insensitive to cholesterol depletion, arguing against a role for conventional ‘lipid rafts’ in the initiation of receptor signalling. Furthermore, decreased mobility was only partially dependent upon the presence of actin which could provide a physical restriction. Importantly, inhibiting Src-family kinase activity, completely abrogated immobilisation. These results are highly suggestive of a previously unrecognised mechanism for the initiation of FcR signalling.
At later stages, receptor-derived signalling leads to the formation of an actin-rich phagocytic ‘cup’. We found that even before a large particle was fully internalised, actin cleared from the base of the phagocytic cup. This clearance was necessary for the internalisation of large particles, as chemically stabilising actin prior to clearance, abrogated internalisation. Actin clearance was shown to be the indirect result of the localised disappearance of phosphatidylinositol 4,5-bisphosphate and the dephosphorylation of tyrosine-phosphorylated proteins. Strikingly, phosphatidylinositol 3-kinase activity was required for both the protein dephosphorylation and for the phosphatidylinositol 4,5-bisphosphate hydrolysis that was responsible for actin disassembly. We propose that actin disassembly is required to recycle actin to the advancing pseudopods, in order to complete phagocytosis.

For many microorganisms, internalisation through phagocytosis means certain death. Obligate intracellular bacteria, such as *Salmonella enterica* serovar Typhimurium however, can readily survive inside host cells. This is achieved through modulation of the host-cell signalling pathways that normally lead to microbial destruction. In S. Typhimurium, a needle-like complex, delivers small protein effectors that aid in the survival of the bacterium. We studied one such effector: SigD, that had been suggested to have phosphatidylinositol phosphatase activity. Indeed, we showed that when the cDNA for SigD was exogenously expressed in mammalian cells, phosphatidylinositol 4,5-bisphosphate was depleted and phosphatidylinositol 5-phosphate was formed. We characterised the physiological effects of this 4-phosphatase activity and furthermore, describe the use of SigD as a research tool for modulating host cell phospholipids.
To the memory of Dr. Nigel Collins. His dedication and passion for Science lives on in those he inspired.
# Table of Contents

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>TABLE OF CONTENTS</td>
<td>V</td>
</tr>
<tr>
<td>LIST OF TABLES</td>
<td>VIII</td>
</tr>
<tr>
<td>LIST OF FIGURES</td>
<td>IX</td>
</tr>
<tr>
<td>LIST OF ACRONYMS AND ABBREVIATIONS</td>
<td>X</td>
</tr>
<tr>
<td>CHAPTER 1</td>
<td>1</td>
</tr>
<tr>
<td>1 INTRODUCTION</td>
<td>1</td>
</tr>
<tr>
<td>1.1 THE INNATE IMMUNE SYSTEM</td>
<td>1</td>
</tr>
<tr>
<td>1.1.1 Introduction</td>
<td>1</td>
</tr>
<tr>
<td>1.1.2 Barrier functions</td>
<td>1</td>
</tr>
<tr>
<td>1.1.3 Cells of the Innate Immune System</td>
<td>2</td>
</tr>
<tr>
<td>1.2 PHAGOCYTOSIS</td>
<td>2</td>
</tr>
<tr>
<td>1.2.1 Introduction</td>
<td>2</td>
</tr>
<tr>
<td>1.2.2 Non-Opsonic Receptors and Their Ligands</td>
<td>3</td>
</tr>
<tr>
<td>1.2.3 Opsonic Receptors and Their Ligands</td>
<td>8</td>
</tr>
<tr>
<td>1.2.4 Interplay Between Receptors</td>
<td>11</td>
</tr>
<tr>
<td>1.3 STRUCTURE AND FUNCTION OF FC-GAMMA RECEPTORS</td>
<td>12</td>
</tr>
<tr>
<td>1.3.1 Introduction</td>
<td>12</td>
</tr>
<tr>
<td>1.3.2 Activating Receptors</td>
<td>12</td>
</tr>
<tr>
<td>1.3.3 Inhibitory Receptors</td>
<td>15</td>
</tr>
<tr>
<td>1.3.4 Non-canonical Fc receptor signalling</td>
<td>15</td>
</tr>
<tr>
<td>1.4 SIGNALLING THROUGH FC-GAMMA RECEPTORS</td>
<td>16</td>
</tr>
<tr>
<td>1.4.1 Initiation of Receptor Signalling</td>
<td>16</td>
</tr>
<tr>
<td>1.4.2 Src Family Kinases</td>
<td>17</td>
</tr>
<tr>
<td>1.4.3 Downstream Kinases</td>
<td>18</td>
</tr>
<tr>
<td>1.4.4 Adaptor Proteins</td>
<td>20</td>
</tr>
<tr>
<td>1.4.5 Small GTPases</td>
<td>23</td>
</tr>
<tr>
<td>1.4.6 The Actin Cytoskeleton</td>
<td>27</td>
</tr>
<tr>
<td>1.4.7 Myosin Family</td>
<td>31</td>
</tr>
<tr>
<td>1.5 MODULATION OF HOST CELL SIGNALLING BY PATHOGENS</td>
<td>35</td>
</tr>
<tr>
<td>1.5.1 Introduction</td>
<td>35</td>
</tr>
<tr>
<td>1.5.2 The Type Three Secretion System</td>
<td>36</td>
</tr>
<tr>
<td>1.5.3 Perturbation of host cell signalling</td>
<td>37</td>
</tr>
<tr>
<td>1.6 IMPETUS</td>
<td>40</td>
</tr>
<tr>
<td>1.6.1 Project #1</td>
<td>40</td>
</tr>
</tbody>
</table>
5 THESIS DISCUSSION AND FUTURE DIRECTIONS ........................................................................................................2

5.1 PROTEIN DOMAINS AS BIOMARKERS FOR LIPIDS ..........................................................................................192

5.1.1 Functional Roles of Lipids ........................................................................................................................192

5.1.2 Phospholipid-Binding Domains ................................................................................................................195

5.1.3 Benefits, Detriments and Alternatives ........................................................................................................195

5.1.4 Quantitative Measurements of Lipids Dynamics .....................................................................................199

5.2 ACTIVATION AND ABROGATION OF FC-GAMMA RECEPTOR MEDIATED SIGNALLING .................................205

5.2.1 Clustering and the Initiation of Phagocytosis .........................................................................................205

5.2.2 Signalling Thresholds and Diffusional Barriers .....................................................................................205

5.2.3 Alternative Mechanisms of Actin Downregulation ..................................................................................209

5.3 MEASURING THE KINETICS OF ACTIN POLYMERISATION .............................................................................216

5.3.1 Introduction ...............................................................................................................................................216

5.3.2 Techniques Used to Acquire Kinetic Data .........................................................................................217

5.3.3 Fluorescent Speckle Microscopy ..............................................................................................................218

5.3.4 Application of FSM to Models of Phagocytosis ......................................................................................218

5.4 MODULATION OF HOST CELL SIGNALLING BY SALMONELLA ............................................................................222

5.4.1 Phospholipid Modulation During Invasion and Potential Roles for PtdIns(5)P ...................................222

5.4.2 Assessment of an Isolated System ............................................................................................................226

5.5 REFERENCES ...................................................................................................................................................229

COPYRIGHT ACKNOWLEDGEMENTS AND DATA ATTRIBUTION ..................................................................................237
List of Tables

TABLE 1: ANALYSIS OF PHOSPHOINOSITIDE CONTENT AFTER SALMONELLA INFECTION .......................................................... 71
TABLE 2: SUMMARY OF FLUORESCENCE RECOVERY AFTER PHOTOLEACHING (FRAP) AT THE UNENGAGED PLASMA MEMBRANE VERSUS THE PHAGOSOMAL CUP ................................................................. 124
TABLE 3: MODULAR INTERACTION DOMAINS USED TO VISUALISE LIPIDS ........................................................................ 196
List of Figures

Figure 1.1: Schematic representation of the scavenger receptor (SR) family ................................................................. 5
Figure 1.2: Structure of human and mouse FC gamma receptors ..................................................................................... 13
Figure 1.3: The activation cycle of small GTPases ........................................................................................................ 24
Figure 2.1: Generation of PtdIns(5)P by SigD. ................................................................................................................. 74
Figure 2.2: Effect of SigD on PtdIns(4,5)P2 distribution and cellular morphology ......................................................... 77
Figure 2.3: Time course of the effects of PtdIns(4,5)P2 depletion on epithelial morphology .......................................... 81
Figure 2.4: Effect of PtdIns(4,5)P2 depletion on F-actin and PtdIns(4,5)P2 distribution .................................................... 83
Figure 2.5: Alternative means of regulating PtdIns(4,5)P2 .............................................................................................. 86
Figure 2.6: Effect of PtdIns(4,5)P2 depletion on junctional integrity ................................................................................ 89
Figure 2.7: Effect of PtdIns(4,5)P2 depletion on anion permeability ............................................................................... 92
Figure 2.8: Effect of PtdIns(4,5)P2 depletion on pH regulation ........................................................................................ 96
Figure 2.9: Phosphoinositide changes induced by SigD .................................................................................................. 114
Figure 2.10: Effect of SigD in renal epithelial cells .......................................................................................................... 116
Figure 3.1: Distribution and photobleaching of PM-GFP in macrophages ...................................................................... 121
Figure 3.2: Photobleaching of PM-GFP at the phagosomal cup ....................................................................................... 126
Figure 3.3: Photobleaching of GFP-TH ........................................................................................................................ 129
Figure 3.4: The effect of actin on the mobile fraction (MF) of PM-GFP in early and formed phagosomes ...................... 132
Figure 3.5: Photobleaching of GPI-GFP at the phagosomal cup ..................................................................................... 135
Figure 3.6: Effect of cholesterol depletion on the mobility of PM-GFP ......................................................................... 138
Figure 3.7: Effect of kinase inhibitors on the mobility of inner and outer membrane leaflet probes .......................... 141
Figure 3.8: Estimation of the time required for the complete phagocytosis of polystyrene beads of different sizes by RAW cells .................................................................................................................. 154
Figure 3.9: Persistence of inner and outer leaflet membrane probes during phagocytosis ............................................. 156
Figure 3.10: Distribution of phospholipid probes in the presence of kinase inhibitors .............................................. 158
Figure 4.1: Clearance of actin at the phagocytic cup ....................................................................................................... 163
Figure 4.2: Localisation of active small GTPases and GEFs during phagocytosis .......................................................... 166
Figure 4.3: Phospholipids regulate the localisation of Tiam1 ......................................................................................... 169
Figure 4.4: Dynamics of phosphotyrosine accumulation during frustrated phagocytosis ......................................... 172
Figure 4.5: Distribution of tyrosine kinases and phosphatases during phagocytosis ..................................................... 175
Figure 4.6: Actin clearance is phosphatidylinositol 3-kinase dependent ....................................................................... 177
Figure 4.7: Actin clearance is evident regardless of actin probe used ............................................................................ 188
Figure 4.8: P-Rex1 is not recruited to forming or internalised phagosomes ............................................................... 190
Figure 5.1: Metabolism of phosphatidylinositol derivatives ......................................................................................... 193
Figure 5.2: Schematic representation of FRAP recovery profiles ................................................................................. 203
Figure 5.3: Localisation of fluorescently labelled Rac during phagocytosis .............................................................. 213
### List of Acronyms and Abbreviations

<table>
<thead>
<tr>
<th>Acronym</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>ADF</td>
<td>actin depolymerising factor</td>
</tr>
<tr>
<td>ADP</td>
<td>adenosine diphosphate</td>
</tr>
<tr>
<td>Arp2/3</td>
<td>actin related protein 2/3</td>
</tr>
<tr>
<td>ATP/ATPase</td>
<td>adenosine triphosphate / adenosine triphosphate phosphatase</td>
</tr>
<tr>
<td>BID</td>
<td>BH3 interacting domain death agonist</td>
</tr>
<tr>
<td>CR</td>
<td>complement receptor</td>
</tr>
<tr>
<td>CTL</td>
<td>C-type lectin</td>
</tr>
<tr>
<td>CTLD</td>
<td>C-type lectin domain</td>
</tr>
<tr>
<td>DAG</td>
<td>diacylglycerol</td>
</tr>
<tr>
<td>DIC</td>
<td>differential-interference contrast</td>
</tr>
<tr>
<td>EEA1</td>
<td>early endosomal autoantigen 1</td>
</tr>
<tr>
<td>ENTH</td>
<td>epsin N-terminal homology</td>
</tr>
<tr>
<td>ESI-MS</td>
<td>electrospray ionisation mass spectrometry</td>
</tr>
<tr>
<td>F - actin</td>
<td>filamentous actin</td>
</tr>
<tr>
<td>FAK</td>
<td>focal adhesion kinase</td>
</tr>
<tr>
<td>FCS</td>
<td>fluorescence correlation spectroscopy</td>
</tr>
<tr>
<td>FRAP</td>
<td>fluorescence recovery after photobleaching</td>
</tr>
<tr>
<td>FSM</td>
<td>fluorescent speckle microscopy</td>
</tr>
<tr>
<td>FYVE</td>
<td>Fab 1, YOTB, Vac 1, EEA1</td>
</tr>
<tr>
<td>G - actin</td>
<td>globular actin</td>
</tr>
<tr>
<td>GAP</td>
<td>guanosine triphosphate phosphatase activating protein</td>
</tr>
<tr>
<td>GBD</td>
<td>guanosine triphosphate phosphatase binding domain</td>
</tr>
<tr>
<td>GDP</td>
<td>guanosine diphosphate</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>GDI</td>
<td>guanosine diphosphate dissociation inhibitors</td>
</tr>
<tr>
<td>GEF</td>
<td>guanine nucleotide exchange factor</td>
</tr>
<tr>
<td>GFP</td>
<td>green fluorescent protein</td>
</tr>
<tr>
<td>GPI</td>
<td>glycosylphosphatidylinositol</td>
</tr>
<tr>
<td>GTP/GTPase</td>
<td>guanosine triphosphate / guanosine triphosphate phosphatase</td>
</tr>
<tr>
<td>HPLC</td>
<td>high performance/pressure liquid chromatography</td>
</tr>
<tr>
<td>Ig</td>
<td>immunoglobulin</td>
</tr>
<tr>
<td>IP₃</td>
<td>inositol 1,4,5-trisphosphate</td>
</tr>
<tr>
<td>IP₄</td>
<td>inositol 1,4,5,6-tetraakisphosphate</td>
</tr>
<tr>
<td>IP₅</td>
<td>inositol 1,3,4,5,6-pentaakisphosphate</td>
</tr>
<tr>
<td>ITAM</td>
<td>immunomodulatory tyrosine-based activation motif</td>
</tr>
<tr>
<td>ITIM</td>
<td>immunomodulatory tyrosine-based inhibitory motif</td>
</tr>
<tr>
<td>LDL</td>
<td>low-density lipoprotein</td>
</tr>
<tr>
<td>LMW-PTP</td>
<td>low-molecular-weight protein tyrosine phosphatase</td>
</tr>
<tr>
<td>LPS</td>
<td>lipopolysaccharide</td>
</tr>
<tr>
<td>LRR</td>
<td>leucine-rich repeat</td>
</tr>
<tr>
<td>LTA</td>
<td>lipoteichoic acid</td>
</tr>
<tr>
<td>MF</td>
<td>mobile fraction</td>
</tr>
<tr>
<td>NHE</td>
<td>sodium / proton exchanger</td>
</tr>
<tr>
<td>NMT</td>
<td>n-myristoyltransferase</td>
</tr>
<tr>
<td>PAMP</td>
<td>pathogen-associated molecular pattern</td>
</tr>
<tr>
<td>PBD</td>
<td>p21 binding domain</td>
</tr>
<tr>
<td>PH</td>
<td>pleckstrin-homology</td>
</tr>
<tr>
<td>PI</td>
<td>phosphatidylinositol</td>
</tr>
<tr>
<td>PI3K</td>
<td>phosphatidylinositol 3-kinase</td>
</tr>
<tr>
<td>PI(4)P-5K</td>
<td>phosphatidylinositol 4-phosphate 5-kinase</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
</tr>
<tr>
<td>--------------</td>
<td>------------</td>
</tr>
<tr>
<td>PI(5)P-4K</td>
<td>phosphatidylinositol 5-phosphate 4-kinase</td>
</tr>
<tr>
<td>PKC</td>
<td>protein kinase C</td>
</tr>
<tr>
<td>PLC</td>
<td>phospholipase C</td>
</tr>
<tr>
<td>PRR</td>
<td>pattern recognition receptor</td>
</tr>
<tr>
<td>PS</td>
<td>phosphatidylserine</td>
</tr>
<tr>
<td>PtdIns(x)P</td>
<td>phosphatidylinositol x-phosphate</td>
</tr>
<tr>
<td></td>
<td>(where x can be 3, 4 or 5)</td>
</tr>
<tr>
<td>PtdIns(x,y)P_2</td>
<td>phosphatidylinositol x,y-bisphosphate</td>
</tr>
<tr>
<td></td>
<td>(where x and y can be any two of 3, 4 and 5)</td>
</tr>
<tr>
<td>PtdIns(3,4,5)P_3</td>
<td>phosphatidylinositol-3,4,5-trisphosphate</td>
</tr>
<tr>
<td>PTEN</td>
<td>phosphatase and tensin-homolog</td>
</tr>
<tr>
<td>ROS</td>
<td>reactive oxygen species</td>
</tr>
<tr>
<td>SCAR</td>
<td>suppressor of cyclic adenosine triphosphate receptor</td>
</tr>
<tr>
<td>SCV</td>
<td><em>Salmonella</em>-containing vacuole</td>
</tr>
<tr>
<td>SFK</td>
<td>Src family kinase</td>
</tr>
<tr>
<td>SHP</td>
<td>Src-homology 2 domain containing (protein) phosphatase</td>
</tr>
<tr>
<td>SHIP</td>
<td>Src-homology 2 domain containing inositol phosphatase</td>
</tr>
<tr>
<td>siRNA</td>
<td>short interfering ribonucleic acid</td>
</tr>
<tr>
<td>SLP</td>
<td>Src-homology 2 domain-containing leukocyte protein</td>
</tr>
<tr>
<td>SPT</td>
<td>single particle tracking</td>
</tr>
<tr>
<td>SR</td>
<td>scavenger receptor</td>
</tr>
<tr>
<td>TEM</td>
<td>transmission electron microscopy</td>
</tr>
<tr>
<td>TIR</td>
<td>toll / IL1 receptor interacting region</td>
</tr>
<tr>
<td>TIR-FM</td>
<td>total internal reflection-fluorescence microscopy</td>
</tr>
<tr>
<td>TLC</td>
<td>thin layer chromatography</td>
</tr>
<tr>
<td>TLR</td>
<td>toll-like receptor</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>T3SS</td>
<td>type III secretion system</td>
</tr>
<tr>
<td>VCA</td>
<td>verprolin-homology, cofilin-homology, acidic</td>
</tr>
<tr>
<td>VHS</td>
<td>Vps27, Hrs, Stam</td>
</tr>
<tr>
<td>WASP</td>
<td>Wiscott Aldrich syndrome protein</td>
</tr>
<tr>
<td>WAVE</td>
<td>Wiscott Aldrich syndrome protein family verprolin homologous</td>
</tr>
<tr>
<td>WIP</td>
<td>Wiscott Aldrich syndrome protein interacting protein</td>
</tr>
<tr>
<td>WT</td>
<td>wild type</td>
</tr>
<tr>
<td>YFP</td>
<td>yellow fluorescent protein</td>
</tr>
<tr>
<td>ZO-1</td>
<td>zona occludens protein 1</td>
</tr>
</tbody>
</table>

Note: Abbreviations for chemical names are given in the relevant Methods section.
Chapter 1

1 Introduction

1.1 The Innate Immune System

1.1.1 Introduction

The human body is rich in favourable environments for colonisation and inhabitation by microorganisms. Such environments are often very stable, having the advantage of homeostatic control to maintain temperature, pH and humidity, often having an abundant supply of organic nutrients. Microorganisms have come to inhabit almost all of these niches despite the varied nature of these environments. Consider for example the arid and exposed nature of the skin inhabited by *Staphylococcus* spp. compared with the moist, warm environment of the mouth favoured by *Lactobacillus* spp. It is perhaps surprising then that despite this sustained assault by pathogenic microorganisms, the human body is not continually crippled by the onslaught of massive and varied infections. The key to this response is the innate immune system. Through a combination of barriers, self recognition and the destruction of non-self antigens the innate immune system gives even newborns a means of defense against a wide range of potentially harmful microorganisms, without having had any previous antigenic exposure.

1.1.2 Barrier functions

Arguably, the first lines of defense against invading microorganisms are the barrier functions of the human body. These primarily comprise the skin and mucosal epithelia. Despite the plethora of microorganisms that normally inhabit these niches, they are kept at bay through a number of mechanisms. Firstly, cells are routinely shed from these layers, providing a disposable barrier. Secondly, secretions from either the barrier cells themselves or other specialised cell types often have microbicidal activity (Flanagan & Willcox, 2009) or at least will immobilise pathogens, allowing them to be physically removed by ciliary action (Chilvers & O'Callaghan 2000). The defenses are not perfect, however, and a combination of these defenses breaking down and the action of microbial systems that serve to compromise the barrier function can result in colonisation and disease (Iwatsuki et al. 2006).
1.1.3 Cells of the Innate Immune System

Barrier function is an important preventive part of the innate immune system, however when the barriers are compromised, invasive pathogens must be recognised and destroyed. There are three main cell types of the immune system that undertake this task; these are macrophages, dendritic cells and neutrophils. These cells play a role in the front line of defense inhabiting the luminal space of epithelial organs, such as the alveolar space occupied by macrophages, the transepithelial space populated by dendritic cells or the endothelial and interstitial space where most neutrophils are found. Upon encountering foreign organisms these cell types will typically be engaged through cell surface receptors and the microbe internalised into a membranous compartment by a process termed phagocytosis. The contents of the now sealed ‘phagosome’ are degraded through fusion of this compartment with vesicles of low pH, which contain lytic enzymes and antimicrobial peptides (Underhill & Ozinsky, 2002 and containing references). These specialised cells not only reduce the microbial load in the tissue by removing pathogens from the milieu, but also play a pivotal role in environmental sampling and antigen presentation, a key part of the adaptive immune system (Medzhitov, 2007), a topic that will not be covered here.

1.2 Phagocytosis

1.2.1 Introduction

While the process of macropinocytosis is often considered “drinking” of the extracellular space, so phagocytosis is considered “eating”. As simplistically explained in the previous section, phagocytosis is the internalisation of foreign bodies. This however is too broad a description as there are several specifics which separate phagocytosis from endocytic or macropinocytic uptake. The two most important are size and receptor dependence. Phagocytosis is typically considered to be a process whereby particles greater than 500nm are internalised. This is a somewhat arbitrary figure, however it serves to delineate cargo that would be too big for endocytic uptake based upon the size of known endocytic machinery (Zimmerberg & Kozlov 2006, Hinrichsen et al. 2006). Phagocytosis is also by definition a receptor-dependent process. This sets it apart from the mostly constitutive uptake through macropinocytosis which, while able to internalise volumes of extracellular fluid that are comparable to a small phagosome (Kerr
& Teasdale, 2009), is not thought to always require a receptor-ligand interaction to initiate uptake.

Phagocytosis can be broadly classified into two types. When an evolutionarily conserved antigen on the surface of a target is recognised directly by a receptor, requiring no involvement of the host immune system, this is termed non-opsonic phagocytosis. The second mechanism relies upon serum peptides called opsonins to ‘prime’ targets before internalisation. Opsonins consist mainly of circulating complement components and serum IgG that, once bound to targets, can be recognised by cognate receptors on phagocytic cells. These two systems and the major receptors for each will be described below.

1.2.2 Non-Opsonic Receptors and Their Ligands

Non-opsonic receptors are those that can initiate phagocytosis when a phagocytic receptor binds directly to an antigen on the target particle. Some of these interactions are the result of an evolutionary response to a particular ligand whilst others recognise molecular ‘motifs’ which can be displayed at the level of short peptide sequences or physical structures (Medzhitov, 2007). These receptors are called Pattern Recognition Receptors (PRRs) and recognise a wide range of non-metazoan motifs, collectively known as Pathogen Associated Molecular Patterns (PAMPs) (Janeway & Medzhitov, 2002). The main families of non-opsonic receptors are described below.

1.2.2.1 Beta-Glucan Receptors

Beta-glucans are polysaccharides found occasionally in bacteria and plants, but most abundantly in fungi where they are major components of the cell wall. These long chain sugars consist of beta-1-3 linked glucose polymers with beta-1-6-linked side chains, a configuration which is not found in animals or plants. Although normally hidden on the inside of the yeast cell wall, beta-glucans are exposed at the bud scar site (Gantner et al. 2005) where they are accessible for receptor binding. Several immune receptors recognise beta-glucans, including scavenger receptors, complement receptors and certain members of the lectin family of receptors. Only the C-type lectin (CTL) Dectin-1, has been shown to elicit an immune response upon binding and internalisation of these particles (Brown, 2006).

In myeloid cells, Dectin-1 (for Dendritic cell-associated C-type lectin-1) is a widely expressed phagocytic receptor. This small transmembrane protein has a single extracellular C-type lectin-
like domain (CTLD) which binds substrate, a transmembrane domain and a single intracellular Immunoreceptor Tyrosine-based Activation Motif (ITAM). Unlike other CTLs Dectin-1 lacks certain cysteine residues adjacent to the CTLD that are thought to mediate dimerisation and as such Dectin-1 likely functions as a monomer.

Upon receptor ligation, the ITAM on the cytosolic side of the protein is tyrosine phosphorylated at a YXXL consensus sequence and phagocytic signalling is initiated (Gantner et al. 2003). Downstream signalling stimulates internalisation of the target and production of anti-microbial reactive oxygen species (ROS) (Underhill et al. 2005) and inflammatory cytokines (Gantner et al. 2003). Importantly, when the receptor function is removed through the use of a blocking antibody (Brown et al. 2002) or in a mouse knockout model (Taylor et al. 2007) both the phagocytosis of yeast particles and the subsequent inflammatory response are inhibited.

A second Dectin-family member was later identified by subtractive cDNA cloning (Ariizumi et al. 2000). Dectin-2 shares many of the same structural features as Dectin-1 and other CTLs however while Dectin-1 binds avidly to Candida yeast bodies, Dectin-2 was shown to preferentially bind to hyphal forms due to subtle structural differences in their respective CTLDs (Sato et al. 2006).

1.2.2.2 Scavenger Receptors

The bacterial surface contains many moieties that are not found in eukaryotic cells. The best studied of these are lipoteichoic acid (LTA) found in Gram-positive bacteria and the lipopolysaccharide (LPS) of Gram-negative cell walls. These lipids can be recognised by certain scavenger receptors (SR) which were first identified through their ability to bind modified low-density lipoproteins in atherosclerotic plaques (Greaves et al. 1998).

The family of SRs has since expanded to include six classes, labelled A-F mainly based upon their tertiary structure and ligand specificity (Krieger, 1997) (see Figure 1.1). There is no obvious domain homology between the receptor classes other than being myeloid expressed transmembrane glycoproteins that bind to modified LDL and polyanionic lipids (Peiser et al. 2001).

The class A scavenger receptors exist in three alternatively spliced forms (collectively termed SRA). The two functional forms at the plasma membrane (SRAI and SRAII), are type II
Figure 1.1: Schematic Representation of the Scavenger Receptor (SR) Family

The Scavenger Receptor (SR) family of transmembrane receptors comprises 6 classes labelled A-F, based upon domain architecture. Examples of typical family members are given below the diagram. Domain abbreviations: CCP: Complement Control Protein, CTLD: C-Type Lectin Domain, SMB: Somatomedin B, EGF repeat: Epidermal Growth Factor-homology repeat. Glycosylation is arbitrarily shown with red ticks. Diagram not to scale.
Figure 1.1 SR Family: Example member: SRA SRECLOX-1CD68SRC1CD36
transmembrane glycoproteins that exist as homotrimers, stabilised by an extended alpha-helical coiled-coil domain. An exofacial collaginous domain is responsible for binding to polyanionic lipids and, in the case of SRA, the recognition of LPS and LTA. Despite containing no canonical phagocytic motifs, SRA has been shown capable of internalising antigenically different strains of bacteria (Thomas et al. 2000, Peiser et al. 2000). Addition of soluble bacterial lipid significantly inhibited this interaction. Furthermore, SRA knockout mice show significantly increased susceptibility to infection by *Staphylococcus* and *Neisseria* spp. (Thomas et al. 2000, Peiser et al. 2002) supporting the idea that SRA acts as a non-opsonic phagocytic receptor for bacterial clearance.

Interestingly, it was found that the absence of SRA also conferred a reduced ability to clear apoptotic cells (Platt et al. 1996, Fukasawa et al. 1996). Anionic lipids are known to be exposed during apoptosis, and these have been shown to be the target of SRA. Further evidence for this interaction was provided by the increased uptake of apoptotic bodies by normally non-phagocytic cells that had been transfected with SRA (Platt et al. 1998). Despite ongoing work, the signalling downstream of the receptor is still unclear, leading to the suggestion that the uptake of apoptotic cells may require an opsonin (Hart et al. 2004) or signalling through a secondary receptor (Peiser et al. 2000) to provide costimulation at the time of binding.

It is interesting that the SRA-mediated phagocytosis of apoptotic bodies signals the release of anti-inflammatory products such as TGF-beta (Fadok et al. 2001), while the phagocytosis of bacterial targets is often accompanied by proinflammatory signals (Peiser et al. 2002). SRA primarily down-regulates inflammation, as shown by the increased production of TNF-alpha in LPS-stimulated SRA knockout mice (Haworth et al. 1997). These data are highly suggestive that the proinflammatory signalling is being regulated through a secondary receptor, although more work is required to better understand SRA-mediated signalling.

### 1.2.2.3 Toll-Like Receptors

The Toll receptor was originally identified in *Drosophila* spp. and was shown to be required for dorsal/ventral polarity during development, as well as having an antimicrobial role (Hashimoto et al. 1988). Shortly after, a family of mammalian homologs were identified and named the Toll-Like Receptors (TLRs). The TLR family members now number 13 (Shi et al. 2009, Kumar et al.
All members of the TLR family, are type I integral membrane glycoproteins, most notable for an extracellular N-terminal domain consisting of approximately 16-28 leucine-rich repeats (LRRs). Each LRR consists of the highly conserved motif “LXXLXLXXN”. The conserved intracellular C-terminal domain is known as the Toll/IL-1 receptor (TIR) domain, so named for its homology to the essential signalling motif of the IL-1 receptor (Beutler et al. 2009).

Given the nature of the LRR, TLRs can bind to a large range of targets including lipids from Gram-positive and Gram-negative bacteria, bacterial DNA, viral and fungal proteins as well as some synthetic compounds (Takeda et al. 2003). The cellular localisation of TLRs seems to be matched to its preferential ligand. Receptors binding to bacterial lipids (TLR1, TLR2, TLR4) or yeast cell wall components (TLR6 and TLR2) are found at the plasma membrane, whereas receptors binding components exposed only after degradation such as bacterial DNA or viral single stranded RNA (TLR9, TLR3/9 respectively), are found in internal compartments. For a more comprehensive review of TLR ligands the reader is directed to several reviews on the topic (Kumar et al. 2009, Akira et al. 2004).

The conjugation of receptor and ligand leads to downstream signalling through the TIR domain that is common to all receptors and is responsible for the recruitment of soluble cytosolic adaptors including MyD88, TRIF, TRAM, MAL and SARM (O’Neill & Bowie, 2007). There is little evidence that these signalling pathways can lead to the physical uptake of bound particles directly, however a downstream effect of receptor ligation is stimulation of an inflammatory response through NF-kappa-B and the type I interferon pathway. This signalling also serves to up regulate scavenger receptors (Doyle et al. 2004), potentially explaining the increased (albeit indirect) phagocytic capacity of cells in the presence of TLRs.

Several TLRs have been localised to forming phagosomes, however the results of TLR genetic knockout are difficult to interpret, as TLR-mediated inflammatory signalling is responsible for stimulating other phagocytic cells. Overall, further work is required to elucidate the roles of TLRs alone and in the context of other phagocytic receptors.

1.2.3 Opsonic Receptors and Their Ligands

Non-opsonic receptors are themselves capable of recognising a wide range of targets. Opsonic receptors, instead utilise an opsonising serum protein to bridge the physical interaction between
host and target. Opsonins have one or more substrate-binding domain and a highly conserved receptor-binding domain. The two main opsonic receptors of the innate immune system are those that recognise immunoglobulin (Ig) and those that recognise complement fragments, notably C3b. Even though both pathways result in the internalisation of opsonised particles, the signalling involved is very different.

1.2.3.1 Complement Receptors

Activation of the complement system is triggered by the cleavage of the C3 protein. The products of this cleavage include the opsonin C3b and several other immune cell-stimulating fragments (Mold, 1999). The C3b fragment binds to and opsonises targets that can then be recognised by complement receptors. Further processing of C3b has been studied in great detail, however this review will only focus on the role of C3b as an opsonin.

Complement (C3b) opsonised particles are bound and internalised through several types of complement receptors (CRs) found on most immune cell types (Underhill & Ozinsky, 2002). CR1 (CD35) is a single pass transmembrane protein with multiple short consensus repeats (SCRs), a large extracellular lectin binding domain which can bind directly to lectins as well as to C3b (Ghiran et al. 2000). The CR2 receptor shares some structural homology with CR1, bearing 15 SCRs, however it is mostly expressed on B cells and is thought to play a role in adaptive immunity, and not as a phagocytic receptor (Gasque, 2004). The complement receptors CR3 and CR4 are structurally quite different from CR1 and CR2. CR3 and CR4 are heterodimeric integrins and, like other integrins, are composed of alpha and beta transmembrane chains. Although the receptors have different alpha chains (CD11b and CD11c respectively), they share a common beta chain (CD18) and both receptors are able to bind to the opsonin C3b (Ehlers, 2000, Ross et al. 1992).

Most of our understanding of complement receptor phagocytosis comes from studies of the CR3 receptor in macrophages and neutrophils. This is due partly to it being the first complement receptor identified, but also because it recognises many microbial ligands other than C3b and can thus act as both an opsonic and non-opsonic receptor. Unlike other phagocytic receptors, ligation of CR3 is not enough to induce phagocytic uptake. Complement-mediated phagocytosis requires an ‘inside-out’ pre-stimulation of the phagocytic cell. In vitro this is typically achieved through the addition of PKC-activating compounds. In vivo the signalling leading to this event is less
clear, although the GTPase Rap1 has been shown to be required for activation of the CR3 beta chain (Caron et al. 2000).

The internalisation of particles by CR3 involves actin-rich membrane ruffling events that are driven mainly by the small GTPase Rho. While a role for Rac and Cdc42 during complement-mediated phagocytosis cannot be dismissed, inhibition of these GTPases does not affect phagocytic efficiency (Caron & Hall, 1998). It is generally thought that particles opsonised with complement ‘sink’ into cells compared to other types of phagocytosis where membrane extensions engulf the particle. It is possible that this observation may be a result of the accompanying membrane ruffling, as Transmission Electron Microscopy (TEM) images have shown both sinking into and extension of membrane as mechanisms of internalisation (Caron & Hall, 1998, Patel & Harrison, 2008).

Unlike phagocytosis mediated through other receptors, purely CR3-mediated internalisation is not accompanied by a respiratory burst and downstream proinflammatory signals. Given the promiscuity of CR3 however, it is likely that many of its ligands will also activate other receptors, leading to the downstream production of inflammatory signals.

1.2.3.2 Immunoglobulin (Fc) Receptors

Immunoglobulins are abundant in blood plasma and serve to opsonise non-self antigens. Immunoglobulins have a highly variable but very specific antigen binding domain (the Fab portion) and a receptor interaction domain that is highly conserved (the Fc portion). There are 5 classes of immunoglobulin in humans, IgA, IgD, IgG, IgE and IgM (for a review see Raghavan & Bjorkman, 1996).

Each of these classes have different roles in innate immunity, however IgG and IgM can opsonise targets and trigger internalisation by phagocytic cells expressing a cognate receptor (Indik et al. 1995b, Uher et al. 1981). It has been conclusively shown that IgG is recognised by the Fc-gamma receptors (see Section 1.3 below); however the receptor specificity is less clear for IgM, which is thought to also be a major opsonin recognised by the complement system (Ogden et al. 2005).

Clustering of Fc-gamma receptors by interaction with a multivalent target initiates signalling involving multiple kinase families and culminating in the activation of the small GTPases Rac
and Cdc42. These actin-regulating GTPases lead to the extension of an actin-rich structure termed the pseudopod which engulfs the target into a largely plasma membrane derived vacuole. Pharmacological inhibition and knockouts of various relevant genes have elucidated the main players in the signalling pathway.

A hallmark of Fc-mediated phagocytosis is an associated inflammatory response. At early stages, phagocytic targets are exposed to antimicrobial reactive oxygen species (ROS) through the action of the NADPH oxidase complex. Secondarily, phagocytic signalling up regulates the production of secreted proinflammatory cytokines that recruit and activate other circulating phagocytic cells to the site of infection (Borroni et al. 2009). Fc receptors are covered in greater detail in Section 1.3 below.

1.2.4 Interplay Between Receptors

Phagocytic receptors are often studied alone, either through a combination of exogenous transfection into non-phagocytic cells or by utilising a specific opsonin under controlled conditions. Care must be taken when comparing this model to a physiological context where phagocytosis is unlikely to be the result of a single receptor type. More likely is a scenario where a variety of receptors (possibly including isoforms of the same receptor and receptors belonging to different families) act in concert to bind and internalise particles. Interestingly, under some circumstances this synergy has been shown to cause a greater response than the sum of the individual signals, suggesting more than simply an additive effect.

For example, in response to beta glucan-coated targets, the Dectin 1 receptor alone stimulates the production of antimicrobial reactive oxygen species. Likewise when stimulated in the same way Toll-Like Receptor (TLR) 4 induces the production of the proinflammatory IL-12 and TNF. When both receptors are stimulated simultaneously, an enhanced stimulation is seen (Gantner et al. 2003), although the mechanism behind this process is not yet fully understood.
1.3 Structure and Function of Fc-gamma Receptors

1.3.1 Introduction

The Fc-gamma receptor family is among the best studied of the phagocytic receptors. A well-established opsonin-receptor interaction allows for a relatively clean study of signalling pathways, without having to pre-activate cells as is necessary for the complement system.

Several receptors bind to the Fc portion of IgG and are thus classified as Fc-gamma receptors. This includes the neonatal IgG transporter FcRn (Raghavan & Bjorkman, 1996) and the phagocytic Fc-gamma receptor. Of the latter family, four main subtypes comprise the Fc-gamma receptors in mice (Nimmerjahn & Ravetch 2008) (see Figure 1.2). Each of these receptors contains at least two classical C2 Ig-like domains on the exofacial side of the protein that bind to the Fc portion of IgG (Williams & Barclay, 1988). Downstream signalling occurs through a consensus tyrosine-based motif that is phosphorylated after receptor crosslinking by antibody. The signalling derived from these receptors is broadly split into two categories; the activating receptors consist of all but one isoform of the Fc-gamma receptors, whilst the final receptor is considered to be inhibitory, and is thought to play an important regulatory role. These two categories will be discussed below.

1.3.2 Activating Receptors

The most studied of the Fc-gamma receptor isoforms is the low affinity Fc-gamma-IIA receptor. This isoform has two extracellular Ig-like domains that are responsible for IgG binding; a transmembrane domain and an intracellular signalling domain bearing an Immunomodulatory Tyrosine based Activating Motif (ITAM). The other activating receptors lack an intrinsic ITAM domain (see Figure 1.2). In these cases, signalling is initiated through an associated gamma-chain that holds the ITAM.

The gamma chain-associated family members are structurally different in other ways. The Fc-gamma-IR has three extracellular C2 (Ig-like) domains, which allows it to interact with ligand with a relatively high affinity compared to the other Fc-gamma receptors (Gessner et al. 1998). Fc-gamma-III family members also have two extracellular Ig-like motifs, however they lack a transmembrane domain and are linked to the extracellular face of the membrane through modification with a glycosylphosphatidylinositol (GPI) anchor. Despite lacking a cytosolic tail
Figure 1.2: Structure of human and mouse Fc gamma Receptors

Schematic representation of inhibitory and activating receptors from human and mouse. All Fc gamma receptors signal bind to IgG through Ig-like domains on their extracellular side (green ovals). Activating receptors signal through an ITAM motif, shown in red which is present either in the receptor itself or in an accessory gamma chain. The sole identified inhibitory receptor signals through an ITIM, shown in yellow. See accompanying text for more details.
Figure 1.2

Activating Receptors

- FcγRIIB: h/m, low-med
- FcγRIIC: h, low-med
- FcγRIID: h/m, low-med
- FcγRIIE: h, low-med
- FcγRIIF: h/m, low-med

Inhibitory Receptor

- FcγRIIB: ITIM

Name: Species: Affinity for IgG:

Name: Species: Affinity for IgG:

Name: Species: Affinity for IgG:

Name: Species: Affinity for IgG:

Name: Species: Affinity for IgG:
or even a transmembrane domain, the Fc-gamma-IIIR is still able to transduce a downstream signal in the absence of other receptors (Park & Schreiber, 1995), although the mechanism by which this occurs is not clearly defined.

As previously alluded to, the key to signal transduction is the ITAM domain of the receptor or associated gamma chain. The signalling domain contains two of these motifs and is highly conserved within Fc receptors as well as components of the B and T cell receptors (Reth, 1989). The domain consensus sequence consists of Tyr-X-X-(Leu/Ile)-X₆₋₈-Tyr-X-X-(Leu/Ile) (with X being any amino acid). Interestingly between the receptors, requirements for these tyrosines in order to complete phagocytosis differ. Both ITAM resident tyrosines are required to transduce a signal from Fc-gamma-IR or Fc-gamma-IIIR (Park et al. 1993), but either is dispensable for Fc-gamma-IIA as long as an upstream tyrosine remains intact (Kim et al. 2001).

Upon crosslinking the activating receptors, the tyrosine residues within the motif become phosphorylated, initiating signalling and leading to the formation of the actin-rich phagocytic cup and ultimately to the internalisation of the opsonised particle.

1.3.3 Inhibitory Receptors

A second type of signal transduction motif exists in the Fc-gamma-IIB receptors, namely the Immunomodulatory Tyrosine based Inhibitory Motif (ITIM). This motif is integral to the Fc-gamma-IIB receptor (Van den Herik-Oudijk et al. 1995) and bears the consensus sequence (I/V/L/S)-X-Y-X-X-(L/V) (Ravetch et al. 2000). Once phosphorylated during receptor signalling (in the same way as the tyrosines in ITAM-bearing receptors) this sequence serves to recruit protein and lipid phosphatases that can then play a role in down regulating the phosphorylation-mediated signalling of the activating receptors (Hunter et al. 1998). As both the activating and inhibitory receptors are expressed in the same cells and bind to the same ligand with comparable affinity, the inhibitory receptors may act to dampen and thus regulate the phagocytic response.

1.3.4 Non-canonical Fc receptor signalling

The dogma in the field suggests that receptors bearing ITAMs will produce activating signals and that ITIM bearing receptors produce inhibitory signals, however there is growing evidence to suggest that this is not the whole story. An example of non-canonical signalling is illustrated by
binding of monomeric IgA to the Fc-alpha-IR which transduces signals through the Fc-gamma-IR associated gamma chain (Pasquier et al. 2005). This has long been shown to be able to induce both anti-inflammatory (van Egmond et al. 2001) and proinflammatory (Kerr et al. 1990) downstream effects. This parallels findings of the ITAM-bearing TCR that can also exhibit both positive and negative signalling, depending upon the strength of ligand interaction (Stefanova et al. 2003).

Furthermore, inhibitory phosphatases involved in Fc-gamma receptor signalling are capable of modulating ITAM signalling even in the absence of inhibitory receptors (Maresco et al. 1999, Kant et al. 2002). This is not proof per se that inhibitory signals do not come from inhibitory receptors, however it suggests that this is not the only mechanism by which regulatory phosphatases can be recruited.

It is important to also consider the potential role of the inhibitory receptors in increasing the physical avidity of the particle interaction even at the cost of a co-incident inhibitory signal. This way, the Fc-gammaIIB receptors might aid in the capture and internalisation of particles without producing too much positive stimulation.

Combined, these data suggest a much more complicated system than simply activating and inhibitory receptors acting in opposite directions. The details of this more complex interaction are yet to be fully explored.

### 1.4 Signalling Through Fc-gamma Receptors

#### 1.4.1 Initiation of Receptor Signalling

The Fc-gamma receptors have affinity for the Fc portion of IgG; however binding of monomeric IgG to a receptor on the cell surface fails to internalise and degrade the immunoglobulin (Jones et al. 1985). It is unclear if this is a result of the generally low affinity of the opsonin/receptor interaction, but more likely is that the conjugation of multiple receptors is required to stimulate uptake. Indeed the heat-induced aggregation of IgG into a polyvalent target stimulates internalisation of the soluble complex through endocytosis (Ukkonen et al. 1986).

When larger particles (over ~500nm) are opsonised with the same ligand they too are internalised. However this occurs in a manner independent of the endocytic machinery requiring
neither clathrin nor ubiquitin (Booth et al. 2002). It still remains unclear as to how the same ligand conjugating the same receptor can produce two mechanistically different results, however it is clear that whether brought about by aggregated IgG, opsonised particles or even sequential receptor crosslinking with primary and secondary antibodies (Kwiatkowska et al. 2003), clustering of the receptors is required for initiation of signalling.

1.4.2 Src Family Kinases

Unlike a number of growth factor receptors that rely upon cross- or auto-phosphorylation events following clustering, the Fc receptors contain no intrinsic kinase activity (Brooks et al. 1989). As such, initiation of signalling relies upon other kinase families. The Src family of non-receptor kinases (SFKs) includes Blk, Fgr, Fyn, Hck, Lck, Lyn, Src, Yes and Yrk (Boggon & Eck 2004). They all share a highly conserved 4-domain structure including a C-terminal protein-kinase domain (contentiously called the SH1 domain), a phosphotyrosine binding SH2 domain, a polyproline binding SH3 domain and a unique domain that differs between family members (sometimes referred to as the SH4 domain) (Abram & Lowell, 2008). Src family members have an N-terminal myristoylation motif that alone is insufficient to anchor them to membranes (McLaughlin & Aderem, 1995, Elliot et al. 1998). The cellular localisation of Src family members is therefore highly dependent upon a secondary lipid modification site, found within the unique (SH4) domain that can be differentially palmitoylated to provide localisation to different membranes in the cell (Sato et al. 2009).

Regulation of Src family kinases is multifaceted, consisting of multiple autoinhibitory interactions between the C-terminal and N-terminal regions (Hubbard et al. 1999). While the mechanism behind initiation of activation after receptor clustering is unclear, it is well established that a direct interaction between SFKs and the Fc receptors significantly increases the activity of the kinase (Ghazizadeh et al. 1994).

The three most common SFKs in phagocytic cells are Lyn, Fgr and Hck, all three of which are primarily associated with the plasma membrane. Upon clustering of Fc receptors by conjugation of a phagocytic particle (see above) SFK phosphorylate the ITAMs in a step thought to be the first event in phagocytic signalling (Cooney et al. 2001). Cells from a Lyn, Fgr, Hck triple-knockout mouse were not completely devoid of FcR-mediated phagocytic capacity, showing reduced internalisation at early time points that recovered to control levels at later times (Fitzer-
Attas et al. 2000). The reason for this residual activity is likely the presence of other SFK present in phagocytic cells, which retain some functional redundancy. Indeed when all SFKs are inhibited using the cell-permeant drug PP1, the ITAM is no longer phosphorylated, phagocytic cups fail to form and as a result, phagocytosis of IgG-coated particles is reduced to the levels of BSA-coated (control) beads (Majeed et al. 2001).

1.4.3 Downstream Kinases

Phosphorylation of the ITAMs on the receptor or associated gamma chain initiates phagocytic signalling by acting as a docking site for soluble proteins containing Src Homology 2 (SH2) motifs (Koch et al. 1991). These domains are ubiquitous in haematopoietic signalling pathways and, among many other proteins, are present in the protein and lipid kinases that are essential for the continuation and completion of phagocytosis (Schaffhausen, 1995).

1.4.3.1 Protein Kinases

While Src-family proteins are thought to initiate phosphorylation of the ITAMs, there is evidence supporting both Src-family kinases and the SH2 domain-containing protein kinase Syk in ITAM phosphorylation (Ibarrola et al. 1997). A role for SFKs upstream of Syk is supported by the previously mentioned SFK inhibitor studies in which treatment with PP1 also inhibits Syk activation. Syk itself is not absolutely required for phagocytosis as shown by the completion of phagocytosis (albeit at a slower rate) in non-phagocytic cells that lack endogenous Syk but have been transfected with Fc receptors (Indik et al. 1995a). Under these circumstances and also in Syk knockout macrophages, distinct defects in gamma chain phosphorylation are seen following Fc receptor clustering (Kiefer et al. 1998), perhaps suggesting a more branched pathway of activation than has been previously considered (Crowley et al. 1997).

What is known however, is that Syk can bind to Fc receptors through its two SH2 domains (Hunter et al. 1999, Chacko et al. 1996) and from there can act as a scaffold to which other proteins can bind and become phosphorylated.
1.4.3.2 Lipid Kinases

The recruitment of kinases to the receptor cluster is not limited to protein kinases. Two main families of phospholipid kinase have been studied extensively with respect to phagocytosis. They differ upon their substrate specificity and so will be dealt with separately.

1.4.3.2.1 Phosphatidylinositol-3 kinases

The phosphatidylinositol-3 kinase (PI3K) family is responsible for the addition of a phosphate to the D3 position of phosphorylated inositol lipids. The three characterised types of PI3K vary in both their substrate preference and mechanism of regulation (Foster et al. 2003). Of primary interest in phagocytosis are the Type I PI3K, which are though to be recruited through the SH2 domains in their regulatory p85 subunits. Upon binding to phospho-peptide sequences, the p85 subunit can physically associate with Syk (Moon et al. 2005). Syk has been shown to phosphorylate p85 (Crowley et al. 1997) leading to the recruitment of the p110 catalytic subunit of PI3K through its SH2 domains (Carpenter et al. 1993). Once recruited the p110 can utilise its preferred substrates: PtdIns(4)P and PtdIns(4,5)P₂, to produce PtdIns(3,4)P₂ and PtdIns(3,4,5)P₃ respectively, and initiate downstream signalling.

Many cellular processes, including survival and development, incorporate parts of the PI3K signalling pathway. This makes the results of genetic knockdown difficult or impossible to interpret. Thankfully, several pharmacological inhibitors of type I PI3Ks exist, notably LY294002 and wortmannin. The former is a reversible competitive inhibitor of the ATP-binding region of PI3K (Vlahos et al. 1994), whereas the latter covalently (and irreversibly) modifies the residue responsible for phosphate transfer (Wymann et al. 1996). When phagocytic cells are treated with LY294002 or wortmannin, initiation of phagocytic signalling, including the formation of an actin-rich cup, is not affected (Cox et al. 1999); however the phagocytic cups stall before the pseudopods fuse (Araki et al. 1996). This finding challenged the idea of a passive ‘zippering’ whereby receptor-opsonin conjugation was thought to suffice to drive formation and closure of a phagocytic cup (Griffin & Silverstein, 1974), and suggested a specific role for PI3K in the completion of phagocytosis.

Several theories have been posited as to why PI3Ks are required, not for the initiation, but for the completion of Fc-mediated phagocytosis. These include roles in the focal delivery of membrane,
recruitment and activation of downstream effectors and production of PtdIns(4,5)P2-based second messengers. It is likely that multiple factors, including all or some of the above, play a role in inhibition of phagocytosis by LY294002 or wortmannin.

1.4.3.2.2 Phosphatidylinositol-5 kinases

A second family of phospholipid kinases utilise the abundant plasma membrane phospholipid PtdIns4P to produce PtdIns(4,5)P2, and have been suggested to play an important role in phagocytosis. The phospholipid, phosphatidylinositol (4,5)-bisphosphate (PtdIns(4,5)P2) can regulate many processes in the cell (DiPaolo et al. 2006). PtdIns(4,5)P2 is not only a direct regulator of many proteins that contain lipid-binding domains (Bottomley et al. 1998), but through the action of the previously mentioned PI3Ks, can also initiate PtdIns(3,4,5)P3-mediated signalling. Furthermore, hydrolysis of PtdIns(4,5)P2 by phospholipase enzymes which are activated during phagocytosis (Liao et al. 1992) produces inositol (1,4,5)-trisphosphate and diacylglycerol, which can activate calcium release from the endoplasmic reticulum and regulate important kinase signalling, respectively (Lennartz, 1999).

Given these numerous signalling pathways, it is perhaps not surprising that during early stages of phagocytosis there is a recruitment of the PtdIns(4,5)P2-producing PI(4)P-5K (Mao et al. 2007), and a subsequent accumulation of its lipid product at the forming phagocytic cup (Botelho et al. 2000). In humans there are three isoforms of PI(4)P-5Ks designated alpha, beta and gamma, each with several functional splice variants. The alpha isoform was initially suggested to provide the PtdIns(4,5)P2 during phagocytosis (Coppolino et al. 2002), however more recent evidence has also indicated a role for PI(4)P-5K gamma.

Genetic knockout or silencing of the alpha or gamma isoforms produces defects in opsonised particle binding and internalisation (Mao et al. 2009). The defects that are seen however, are not only the result of less PtdIns(4,5)P2 at the phagocytic cup. The PI(4)P-5Ks can also regulate the actin cytoskeleton directly through interactions with WASP and the small GTPases Rac and Cdc42, and it is likely that a combination of these two effects causes inhibition of phagocytosis.

1.4.4 Adaptor Proteins

The previously mentioned components of the signalling pathway are all ‘active’ in the sense that they involve catalytic activity of some sort. These enzymes can initiate signalling (such as the
Src and Syk family kinases), but rely upon the formation of a molecular scaffold to propagate signals. This scaffolding comes about with the recruitment of adaptor proteins, which themselves have no intrinsic activity but through common protein-protein and protein-lipid interaction domains serve to couple upstream to downstream signals (Morton & Campbell, 1994, Kuriyan & Cowburn, 1997).

Many proteins contain these interacting domains and so only the adaptors shown to play a role in phagocytosis will be covered here.

1.4.4.1 Gab and Grb

The Gab family, consisting of mammalian Gab1, Gab2 and Gab3, only share between 40-50% sequence similarity, but nevertheless have comparable domain structure (Gu & Neel, 2003). An N-terminal pleckstrin-homology domain serves to interact with triply phosphorylated phosphoinositides, while at least two proline-rich motifs serve as interaction platforms for SH3 domain-containing proteins (Lock et al. 2000). Furthermore, Gab proteins can be tyrosine-phosphorylated at multiple sites (Shinohara et al. 2001, Lehr et al. 1999, Lehr et al. 2000), allowing further interaction with SH2 domain-containing proteins. Interestingly, two such phospho-tyrosines can act to stably associate Gab with another adaptor protein, GRB2 (Lock et al. 2000, Schaeper et al. 2000). This interaction was the basis for the original identification of the GRB2-Associated Binder (Gab) (Holgado-Madruga et al. 1996) and has since provided clues to the many interacting partners of the Gab family.

Fc-mediated phagocytosis is a prototypical phosphotyrosine driven event and it should therefore not be surprising that Gab2 is recruited to the forming phagocytic cup and remains associated with the closed phagosome for several minutes (Gu et al. 2003). Curiously, this seems to both require PtdIns(3,4,5)P₃ and stimulate further production of the same lipid, suggesting a system of positive feedback, which has been proposed to act as a threshold for completion of phagocytosis (Beemiller et al. 2010, Swanson, 2008). Indeed, studies in cells from Gab2 knockout mice show reduced ability to complete phagocytosis (Gu et al. 2003), with cups morphologically similar to those seen when PtdIns(3,4,5)P₃ production is inhibited (Araki et al. 1996).
1.4.4.2 Crk Family

The Crk family of adaptor proteins has 3 members, highly conserved in their shared domains (Galletta et al. 1999). The Crk family members have a single SH2 domain and varying numbers of SH3 domains that allow for protein-protein interactions with a wide range of proteins, including other adaptors (Sakkab et al. 2000), nucleotide exchange factors (Hasegawa et al. 1996), protein kinases (Ren et al. 1994) and transcription factors (Rhodes et al. 2000) among others (Feller et al. 2001).

In *Caenorhabditis elegans* the CrkII homolog CED-2 was shown to mediate clearance of apoptotic cells through interactions with the nematode homologs of DOCK180 and Rac (Wang et al. 2003). In mammalian phagocytic cells, specifically macrophages, CrkII is the predominant species with lower levels of CrkI and CrkL expression. CrkII contains the conserved N-terminal SH2 domain whereby the protein is recruited to the receptor complex and two C-terminal SH3 domains that mediate downstream interaction (Matsuda et al. 1992). Mutation of key phosphotyrosine or proline-binding residues in these domains, or knockdown with siRNA prevents the recruitment of the Rac exchange factor DOCK180 to the phagocytic cup, resulting in severe inhibition of Fc-mediated phagocytosis (Lee et al. 2007).

1.4.4.3 SLP76 Family

The Src-homology 2 domain-containing Leukocyte Protein of 76kDa (SLP76) is expressed in T cells and myeloid cells, and is the prototypical member of the SLP76 family of adaptor proteins. Similarity between family members is based upon a conserved domain structure consisting of an N-terminal region that bears multiple tyrosine-phosphorylation sites, a central proline-rich region and a C-terminal SH2 domain (Wu et al. 2004).

Upon clustering and activation of ITAM-bearing receptors it is thought that Syk (Bonilla et al. 2000) or its homolog in T-cells, ZAP70 (Bubeck-Wardenburg et al. 1996) will phosphorylate the N-terminal tyrosines in SLP76, providing a docking site for other SH2 domain-containing proteins. Through the poly-proline and SH2 domains, SLP76 is capable of coupling a large number of downstream proteins including phospholipase C gamma isoforms (Zhang et al. 1998), the Rac exchange factor Vav (Tuosto et al. 1996) and the adaptor protein Nck, which can
indirectly induce actin polymerisation through interaction with the p21-activated protein kinase (Bubeck-Wardenburg et al. 1998).

During Fc-mediated phagocytosis, SLP76 was shown to be recruited to phagocytic cups and subsequently phosphorylated (Coppolino et al. 2001). In the absence of SLP76 however, phagocytosis occurs normally, with only a small defect in phospholipase-C activation (Bonilla et al. 2000). This finding was thought to be a result of the overlapping functions of other SLP76 family member; however further work in macrophages lacking both SLP76 and SLP65 showed similar results, with no inhibition of growth, phagocytosis or downstream reactive oxygen production (Nichols et al. 2004).

1.4.5 Small GTPases

1.4.5.1 Introduction

The Rho family of proteins is part of the Ras superfamily of small GTPases and consists of 14 members in three subfamilies, the Rho, Rac and Cdc42 proteins. The family members are structurally similar GTPases of 20-30 kDa and are responsible for regulating many cellular processes, from membrane traffic to transcriptional control (Bishop et al. 2000). Rho-family GTPases are considered the classic ‘molecular switch’ (see Figure 1.3), alternating between their active and inactive state when bound to GTP and GDP, respectively (Etienne-Manneville et al. 2002). Intrinsic GTPase activity is typically low (Paduch et al. 2001) and so regulation occurs through the binding of three types of ancillary proteins. The GTPase-activating proteins (GAPs) bind to the small GTPases and stabilise catalytic residues in the active site (Rittinger et al. 1997), increasing GTPase activity 4-8 orders of magnitude (Schefzkek et al. 1997). While the action of GAPs serves to inactivate small GTPases, another family of proteins, the Guanine nucleotide-Exchange Factors (GEFs) enhance the native (but again, slow) dissociation of GDP from the nucleotide binding pocket (Worthylake et al. 2000), allowing insertion of a new GTP, activation of the GTPase and subsequent stimulation of the downstream GTPase effector proteins.

The third main group of regulatory proteins bind only to the GDP-bound small GTPases and counteract the activity of the GEFs. Because of this activity they were named GDP-Dissociation Inhibitors (GDIs). A fundamental role of the GDIs is masking the lipid tail present on most small GTPases (Roberts et al. 2008). This way the GDIs inhibit activity in two ways; by preventing
Figure 1.3: The Activation cycle of small GTPases

The small GTPases of the Rho-superfamily represent the classic ‘molecular switch’. They alternate between an active and inactive state when bound to GTP and GDP respectively (the nucleotide is shown in red). When GTP bound, the small GTPases are membrane associated and can activate downstream effectors (see accompanying text). Inactivation of the small GTPases occurs through the GTPase activating proteins (GAPs) which indirectly stimulate the hydrolysis of GTP. Once deactivated, GDP dissociation inhibitors (GDIs) prevent activation of the small GTPases and shroud the acyl tail, solubilising the protein. Activation of the small GTPases occurs through the action of Guanine-nucleotide Exchange Factors (GEFs) which aid in the GTP loading of the GTPases.
Figure 1.3
their activation by GEFs and by solubilising the membrane-associated GTPase, physically
separating the enzyme from its substrate.

The earliest identified roles of the small GTPases Rac, Cdc42 and Rho were in organisation
of actin-rich structures within the cell in response to external stimuli (Nobes & Hall, 1995). The
similarity between membrane ruffles and the phagocytic cup was suggestive of a role for these
small GTPases; indeed all three family members are recruited to the forming phagocytic cups of
different receptors (Caron & Hall, 1998). Furthermore, many studies using dominant-negative
constructs, clostridial toxins and RNA silencing have shown significantly inhibited phagocytosis
in the absence of one or more of these family members (Cox et al., 1997, Hackam et al. 1997).
Even though the specific roles of Rho, Rac and Cdc42 during Fc receptor mediated phagocytosis
remain incompletely understood, certain conclusions can be drawn based upon the previously
mentioned experimental techniques.

1.4.5.2 Rac and Cdc42

Crosslinking Fc-gamma receptors leads to an increase in activated Rac and Cdc42 (Forsberg et
al. 2003). However, understanding of the specific roles of these proteins has almost exclusively
been investigated using dominant-negative constructs (Cox et al. 1997), occasionally making
results difficult to interpret. Inhibiting either Rac or Cdc42 in this way leads to a significant
decrease in phagocytosis (Massol et al. 1998).

The type of actin structures produced in response to receptor crosslinking varies, being primarily
filamentous when Cdc42 is active and mostly lamellar in the presence of active Rac (Caron &
Hall, 1998). The specific role of these family members is unknown; however it is clear that not
only are they activated in separate ways (Patel et al. 2002), but the downstream pathways
activate different effectors (Hoppe & Swanson, 2004). This was perhaps most obvious in
experiments with artificially clustered Cdc42 and Rac that showed that while actin is
polymerised in both cases, only in the latter case would phagocytosis be complete (Castellano et
al. 2000).

These findings support the idea of Cdc42 playing a role in early actin events, such as the
‘probing’ of opsonised particles (Castellano et al. 1999, Kress et al. 2007) and Rac playing a
later role in cup progression and completion through essential interactions with downstream factors such as lipid kinases (Hartwig et al. 1995) and actin-related proteins (Miki et al. 1998).

1.4.5.3 Rho

Rho-family proteins are structurally similar to Rac and Cdc42 and are also recruited to the phagocytic cup of IgG-opsonised particles. Despite this, Rho has been shown to be dispensable for Fc-mediated phagocytosis in several studies. In experiments using the C3 exotoxin of Clostridium botulinum, which selectively inhibits Rho, actin polymerisation at the phagocytic cup of IgG coated particles was shown to be normal, as were the kinetics of completion (Caron & Hall, 1998, May et al. 2000). It is not surprising that Rho plays a much more important role in complement-mediated phagocytosis, which is morphologically associated with contractile actomyosin structures. This is in contrast to the filopodial and lamellipodial structures typical of Cdc42 and Rac respectively (Hall, 1998, Ridley & Hall, 1992, Ridley et al. 1992, Kozma et al. 1995).

Despite this, there is some evidence to suggest a subtle role for Rho in the initial steps of receptor clustering and the downstream tyrosine phosphorylation events that lead to successful completion of phagocytosis (Hackam et al. 1997). Inherent phagocytic competence or the difficulties in working with and delivering the C3 exotoxin could go towards explaining this dichotomy and as such this remains a topic of study.

During initiation of phagocytosis, the small GTPases can be activated when brought into the vicinity of GEFs at the phagocytic cup. Once GTP-loaded they are more stably membrane-associated; however they contain no endogenous actin nucleation or polymerisation-promoting domains and so rely upon downstream proteins to induce the formation of the actin-rich phagocytic cup.

1.4.6 The Actin Cytoskeleton

1.4.6.1 Actin Nucleation

The actin cytoskeleton has myriad roles, having long been shown to play critical roles in migration, cell division and adhesion (Le Clainche & Carlier, 2008), and only more recently in endocytosis (Robertson et al. 2009) and nuclear gene transcription (Zheng et al. 2009). One of
the defining features of phagocytosis is the actin-mediated formation of a phagocytic cup. It is worth noting that the formation of an organised cup is completely abrogated when actin polymerisation is inhibited (Araki et al. 1996).

Signal transduction from the GTPases Rac and Cdc42 to the nucleation and polymerisation of actin occurs through the WASP/WAVE proteins and the Actin-Related Protein 2/3 (Arp2/3) complex. The ubiquitously expressed N-WASP is thought to be normally autoinhibited by an intramolecular association between its GTPase-binding domain (GBD) and a verprolin homology, coflin-homology, acidic (VCA) domain (Stradal et al. 2004), which is destabilised upon binding of GTP-bound Cdc42 and charged phospholipids, respectively to the GBD and basic domains (Rohatgi et al. 2000). This is an over-simplistic model however, as a family of WASP-interacting proteins (WIPs) bind to autoinhibited WASP (Martinez-Quiles et al. 2001) and can alter the requirements for activation of the complex (Ho et al. 2004, Derivery & Gautreau, 2010).

Even though there is some evidence to suggest that Rac plays a role in the previously described scheme (Tomasevic et al. 2007), the better understood role for Rac is thought to be through the WASP family verprolin homologous (WAVE) proteins, also known as the Suppressor of cAMP receptor (SCAR). The main difference in this pathway is that the Rac effector WAVE does not contain a GBD (Pollard et al. 2003) and is considered to be constitutively active in the cytosol (Machesky et al. 1999). In order to stimulate the formation of actin structures, WAVE likely interacts through a multimolecular PIR121/Nap1/Abi/HSPC300 complex (Gautreau et al. 2004), of which PIR121 is likely to bear the Rac interaction motifs (Kobayashi et al. 1998).

The complex formed by active GTPases binding to WAVE or WASP is still insufficient to nucleate and polymerise actin. That function requires the action of the seven protein, Actin-Related Protein 2/3 (Arp2/3) complex. Two subunits of this complex, Arp2 and Arp3 bear striking resemblance to actin monomers themselves (Volkmann et al. 2001) and can serve as a seed for polymerisation by lowering the energy requirement for spontaneous nucleation (Kelleher et al. 1995). The remaining subunits of the Arp2/3 complex are responsible for stabilising the complex and interacting with the exposed VCA domains of active WASP/WAVE (Suetsugu et al. 2001).
Early experiments with WASP-deficient leukocytes provide evidence of a critical role for these adaptors in bridging signalling from Rac and Cdc42 to the polymerisation of actin and subsequent formation of the phagocytic cup. When challenged with IgG-opsonised particles WASP-null cells fail to form actin-rich phagocytic cups and are incapable of internalising particles (Lorenzi et al. 2000), supporting the idea of actin polymerisation as the driving force behind pseudopod formation and extension (Sechi & Wehland, 2000).

In other systems, actin nucleation can be spontaneous (albeit energetically unfavourable (Sept & McCammon, 2001)), mediated by Arp2/3 as described above or through Spire (Quinlan et al. 2005), Diaphanous-related formins (Goode et al. 2007) or the recently identified JMY53 (Zuchero et al. 2009). Interestingly however, if components of the Arp2/3 complex are removed or disrupted, formation of actin-rich phagocytic cups is completely abrogated (May et al. 2000). These findings are suggestive of a lesser (or potentially indirect) role for these other nucleators in Fc receptor-mediated phagocytosis. Interestingly, this is not the case for phagocytosis mediated through other receptor types (Colucci-Guyon et al. 2005).

1.4.6.2 Actin-Binding Proteins

Nucleation of actin filaments is only the first step in the formation and stabilisation of a filamentous actin network. Many proteins play a role in regulating forming or pre-formed filaments (Winder & Ayscough 2005). This overview will focus only on the major actin-binding proteins that have been studied in the context of phagocytosis.

1.4.6.2.1 Gelsolin

A well-studied family of actin-binding proteins is the Gelsolin family, characterised by the presence of multiple Gelsolin (G) domain repeats which are present in varying numbers between family members (Silacci et al. 2004). In the presence of calcium, this usually globular protein unfolds and exposes actin-binding sites through its six G domains. After binding to filaments, gelsolin will lead to the destabilisation and severance of filaments, but is thought to remain attached to act as a capping protein (Sun et al. 1999).

Work conducted in a knockout mouse model suggested an important role in macrophage ruffling and phagocytosis for the gelsolin family member CapG, whose elimination impaired phagocytosis by around 50%. Interestingly no such effect was seen if gelsolin itself was absent
(Witke et al. 2001), provoking important questions regarding the differential regulation of two highly similar family members. Remarkably, in neutrophils, gelsolin was required for efficient Fc mediated phagocytosis, which was abrogated in the knockout cells (Serrander et al. 2000).

1.4.6.2.2 ADF / Cofilin

The Actin-Depolymerising Factor (ADF)/Cofilin family of proteins are actin filament destabilising proteins that have been implicated in the rapid formation of the sort of branched actin networks that are seen during filopodial dynamics (Fass et al. 2004) and chemotactic migration (Gupton et al. 2005). Cofilin binds to both ADP F-actin and ATP F-actin, although the former occurs with a greater affinity, thus the older filaments are more prone to interaction with this protein. Binding produces a twist in the filament of approximately five degrees per subunit, which alters its thermodynamic stability and thus induces both depolymerisation and severing (McGough et al. 1997).

When cofilin is inhibited through the use of blocking antibodies or genetic silencing, a significant inhibition of complement-mediated phagocytosis is seen (Nagaishi et al. 1999). While this is perhaps not surprising given the complex regulation of cofilin downstream of Rac (Edwards et al. 1999), Cdc42 (Dan et al. 2001) and Rho (Ohashi et al. 2000), there has been no evidence of inhibition yet published for Fc receptor-mediated phagocytosis.

1.4.6.2.3 Profilin

Actin exists in equilibrium between its filamentous form and a pool of monomers. While many proteins regulate actin at the level of the filament (as above), regulation also occurs at the level of the actin monomers. In the same way that Rho family GEFs aid in the exchange of GDP for GTP, so do the profilin family of actin-binding proteins bind to ADP-actin and aid in the exchange for ATP (Goldschmidt-Clermont et al. 1992). This serves to prime actin monomers for addition to a filament. In several cases where large and rapid actin rearrangements are required, profilin is recruited through complementary signalling pathways to enhance the local pool of polymerisable (that is, ATP-bound) actin (Le Clainche & Carlier, 2008).

During phagocytosis, profilin is recruited to the phagocytic cup in a manner dependent upon a complex downstream of receptor signalling containing the scaffolding adaptors Ena/VASP (Copollino et al. 2001). The exact role of profilin in a mammalian system has not yet been fully
elucidated. Studies of knockouts in other model systems show conflicting results, with both increased phagocytosis in *Drosophila melanogaster* (Pearson *et al*. 2003) and decreased pinocytic fluid uptake in *Dictyostelium discoideum* (Seastone *et al*. 2001). These different results may represent structural differences in the protein itself or, alternatively, different modes of regulation. Regardless, further study of this protein’s role in mammalian cell phagocytosis would be required to draw further conclusions.

In most cases it is difficult to elucidate the role of individual actin-binding proteins in the regulation of actin. This is mainly due to the very fine control that is maintained over cellular actin, which if perturbed by experimental techniques can have unexpected effects. A good example of this are actin-severing proteins that on one hand are thought to depolymerise actin filaments, but under the right conditions can also create free barbed ends and induce large actin rearrangement.

1.4.7 Myosin Family

1.4.7.1 Introduction

One group of actin interacting proteins that have been intensely studied in the field of phagocytosis is the myosin family. Myosin-family proteins are the canonical ‘molecular motors’, best known for their role in muscle contraction. This large family of over 20 classes of proteins (Berg *et al*. 2001) also plays many important roles in non-muscle cells. Aside from the well published roles in organelle and cargo trafficking (Tuxworth & Titus 2000) myosin proteins have been implicated in establishing cell polarity (Yin *et al*. 2000) stimulating actin polymerisation (Evangelista *et al*. 2000) and in regulating signal transduction (Bahler *et al*. 2000).

The myosin family is typified by the presence of three domains. A globular head domain is responsible for binding to actin filaments and the ATP-dependent motor properties. A linker region is responsible for protein-protein interactions and a tail domain bears class-specific regulatory properties (Krendel *et al*. 2005). The myosin family was originally split into ‘conventional’ and ‘unconventional’ myosins based upon dimerisation state and divergence in the tail domain. This nomenclature is somewhat archaic however, as both groups have been shown to contain diverse tail function and both groups have members that can act as monomers
as well as dimers. Regardless, this review will first focus on evidence for localisation to phagocytic cups and secondly upon the potential roles of myosins during phagocytosis.

1.4.7.2 Localisation to Phagocytic cups

Many myosin-family members have been localised to phagocytic cups during phagocytosis. This includes myosin IC, non-muscle myosin II, and myosins V, VII and X among others (Swanson et al. 1999, Mooseker & Cheney, 1995, Spudlich, 1994, Cox et al. 2002). In most cases the mechanism of recruitment to phagocytic cups is unclear. Nevertheless, some specific mechanisms of recruitment have been identified.

Myosin I for example, contains an SH3 domain as well as a predicted PH domain (Bement et al. 1994, Brzeska et al. 2008). These modular protein-protein and protein-lipid interaction domains can interact with signalling adaptors and acidic phospholipids respectively. These are two components that are present in abundance during phagocytosis and combined with the presence of actin, could recruit myosins to their site of action.

Myosin X is unique since, unlike other myosins, it contains PtdIns(3,4,5)P3-binding PH domains (Berg et al. 2000). As PtdIns(3,4,5)P3 is only produced at the later stages of phagocytosis, this provides a means of recruitment to phagocytic cups in a spatially and temporally regulated manner. Furthermore, abrogation of PtdIns(3,4,5)P3 production prevents recruitment of Myosin X, suggesting a limited role for other interactions in its recruitment (Cox et al. 2002).

While these mechanisms of recruitment have been shown within the context of Fc-mediated phagocytosis, there are still many myosin proteins and classes whose mechanism of recruitment is as yet unknown. There are many possibilities for recruitment including the previously mentioned domains as well as the possibility of recruitment, through adaptors or myosin light chains interacting with the phagocytic signalling complex.

1.4.7.3 Roles for myosins

The localisation of myosins to phagocytic cups is suggestive but not indicative of a role in phagocytosis. The functional role of different myosins during phagocytosis has been a topic of much study and debate. Most work has tried to elucidate functional roles by looking at the
effects of inhibition. The following sections describe some of the proposed roles for myosin family members during phagocytosis.

**Myosins as structural proteins:** Specialised myosins found in muscle as well as those involved in vesicle transport are processive motors. Many other myosins are non-processive and this property allows them to act as structural-proteins to crosslink actin filaments. The polymerised actin at the phagocytic cup is highly organised and this requires filament crosslinking to stabilise the structure. Crosslinking requires at least two actin-binding domains. The myosin head domain is known to bind actin filaments and so satisfies half of this requirement. Dimerisation and the presence of a second actin-binding domain have both been shown to occur in myosin family members (Kalhammer & Bahler, 2000, Geli et al. 2000), fulfilling the second requirement for this structural role.

The idea of myosin as a structural protein is supported by experiments with ML7, a myosin light-chain kinase inhibitor. The effect of this drug is to inhibit the activation of the myosin regulatory light chain and thus inhibiting myosin function. When this drug is used, the resulting phagocytic cups are loose and malformed when viewed ultrastructurally. This is what would be expected based upon the inhibition of crosslinking, as the cups that form are not closely apposed to the target particle. Functionally these cups are insufficient for the completion of phagocytosis as they are unable to completely engulf the target particle (Swanson et al. 2002). Interestingly this observation also provides further evidence against the simple ‘zippering’ model originally proposed by Griffin & Silverstein (1974). Here, interaction between the opsonin and receptor should not be inhibited, suggesting that there is an underlying structural requirement for phagocytosis.

**Myosins to generate force:** Another explanation for the phenotype seen with the non-specific inhibition of myosins is that they are required to generate force (instead of passively crosslinking filaments). The requirement for force generation has been suggested to play two main roles during phagocytic internalisation:

The loose cups seen with myosin inhibition may be the result of a lack of a circumferential force during the growth of the cup. This ‘squeezing’ force is an attractive hypothesis as it explains the deformation seen during the internalisation of malleable targets (Araki et al. 2003). In lieu of the zippering model, the presence of this force also suggests a mechanism behind the purse string-
like closure of the phagocytic cup (Swanson et al. 1999). During phagocytosis the diameter of the leading edge of the phagocytic cup increases to that of the maximum diameter of the target, then reduces in size until closure of the cup. The deformation of malleable targets suggests this force is present throughout the process (Araki et al. 2003) and so, such a force would require tight regulation so as not to preclude early expansion of the cup. This regulation is perhaps mediated by the temporal recruitment of different myosins during phagocytosis, a suggestion which is supported by immunofluorescence studies (Diakonova et al. 2002, Swanson et al. 1999).

A second potential role for force generation is in the physical internalisation of particles. During endocytosis, a nascent endosomal ‘pit’ is formed at the plasma membrane, which invaginates and gets internalised to form an endosome. Recently, a role for actin in certain types of endocytosis has been proposed (Kaksonen et al. 2006). The actin is thought to provide the pulling force to drive the invagination of the nascent pit. Endocytosis in *Saccharomyces cerevisiae*, also requires the action of yeast myosins III and V (Barker et al. 2007). It is conceivable that actin and myosin are also performing this second role during phagocytosis, providing the force to physically internalise target particles.

In support of this idea, a role for the *Dictyostelium* unconventional myosin VII has been suggested in generating the pulling force required to internalise target particles into the cell body (Titus, 1999). Whilst inhibition of this isoform drastically inhibited internalisation of particles, studies on this protein are yet to be repeated in a mammalian system (Tuxworth et al. 2001). This work also highlights the importance of specific inhibition. If a combination of circumferential and pulling forces are required, then broad spectrum inhibitors will not be able to distinguish the two.

**Other roles for Myosins;** There are several alternative roles for myosins that could explain their inhibition leading to a phagocytic defect. The focal delivery of membrane is known to be required for the successful completion of phagocytosis, especially with larger particles (Niedergang et al. 2003). One of the well established roles for myosins is in the trafficking of membrane vesicles (Tuxworth & Titus, 2000). It is therefore possible that broadly inhibiting myosins could inhibit the delivery of membrane to the forming phagocytic cup. Although myosins have been proposed to play a role in transport of the phagosome away from the site of
internalisation (Diakonova, 2002), a role in delivery of membrane to the phagosome is speculation at this time.

Little is known about the nature of the interaction between actin in the extending pseudopod and the membrane of the phagocytic cup. Current thinking suggests that this interaction is regulated through the WASP/WAVE proteins (see Section 1.4.6 and Takenawa, 2007). In specialised epithelium, myosin Ia has been shown to bridge actin filaments to acidic phospholipids (Hayden et al. 1990). This is critical in the maintenance of the brush border, as knockout of this myosin isoform destroys the polarised morphology of the epithelial layer (Tyska et al. 2005). In phagocytic cells, myosin Ia may be playing a similar role in order to stabilise the forming phagocytic cup; however such a role would have to be tightly regulated given the rapid and dynamic nature of phagocytosis. A similar role has been proposed for the PH domain-containing myosin X. Although a functional role for this myosin has not been resolved, it has been suggested that through its PH domains, myosin X serves to crosslink PtdIns(3,4,5)P_3 to the actin cytoskeleton at the phagocytic cup. This would act to couple movement of the underlying cytoskeleton to membrane movement at the cup, although this hypothesis requires further investigation.

At present there are too few selective inhibitors to study the roles of individual myosins during phagocytosis. This section has described several published roles and conjectured as to potential functions of myosins during phagocytosis. Characterisation of myosins has come a long way, however many are still without identified binding partners or functional roles (Nambiar et al. 2010) and as such work focused on specific myosin classes during phagocytosis will greatly aid in our understanding of the function of these important proteins.

### 1.5 Modulation of Host Cell Signalling by Pathogens

#### 1.5.1 Introduction

Phagocytosis is mechanistically a marvel of host adaptation to challenges by pathogens. This system can recognise, internalise and destroy a wide variety of potentially pathogenic organisms. This however, provides a strong selective pressure under which the evolution of microorganisms is forced. Indeed, many bacteria have evolved methods to subvert the previously described
signalling pathways that could otherwise lead to their internalisation and destruction. The difficulty lies in how to manipulate the host cell signalling pathways from outside that same host cell, when proximity entails the danger of being internalised and degraded. This problem is elegantly solved by some pathogens through the use of a specialised secretion complex that allows for delivery of effector proteins directly into the host cell.

1.5.2 The Type Three Secretion System

The Type I and Type II bacterial secretion systems allow direct or indirect translocation respectively, of proteins into the extracellular space. Among other functions, these systems are utilised to secrete toxins, damage host barriers, digest complex nutrients, scavenge other factors and maintain the bacterial cell wall (Wickner & Schekman, 2005). Most extracellular factors are of limited use when attempting to perturb host cell signalling. As such, many bacterial pathogens utilise a Type III secretion system (T3SS) to bypass the extracellular milieu and deliver effector proteins directly into a host cell. From here the effectors can elicit their response and modulate host cell signalling directly (Galan & Wolf-Watz, 2006).

The T3SS is found in many bacterial pathogens, including the three subtypes of human pathogenic *Yersinia* spp., *Shigella* spp., *Salmonella* Typhimurium, enteropathogenic and enterohemorrhagic *Escherichia coli* and *Pseudomonas aeruginosa*, (Hueck et al. 1998). Contentiously thought to have evolved from a flagellum-like structure (Saier et al. 2004), the secretion apparatus consists of a pair of multi-ring transmembrane complexes that span the inner and outer membranes of the bacterium, and a needle-like column that protrudes from the cell (Galan & Wolf-Watz, 2006). Contrary to initial suggestions (Hoiczyk & Blobel, 2001), the needle itself is not sufficient to transport effector proteins into host cells (Hakansson et al. 1996). This requires additionally, the secretion of translocator proteins that form a ‘docking’ complex for the tip of the needle. This aids the passage of effectors directly into the host cell cytosol (Tardy et al. 1999).

Through this and other similar systems (Christie et al. 2005) bacteria can deliver a wide range of host-modulating proteins to perturb host cell signalling and ultimately improve their own survival.
1.5.3  Perturbation of host cell signalling

As early as 1956, microbiologists demonstrated that pathogenic bacteria are able to resist phagocytic uptake in a contact-dependent manner (Bacon & Burrows, 1956). Since the identification and characterisation of the T3SS, a better understanding of the mechanism of delivery has allowed the study of specific effectors and their role in the modulation of host cell signalling.

Much work has focused upon *Yersinia* as a model organism. These obligate extracellular bacteria cannot survive inside a phagocytic cell and thus, have evolved to use their T3SS to inhibit internalisation through phagocytosis. The mechanism of inhibition is focused upon preventing the host cell from forming a phagocytic cup. In other bacteria, diverse mechanisms of host modulation exist to aid in bacterial survival. *Yersinia* specifically targets portions of the phagocytic signalling pathway focused on during this thesis. Therefore, by way of example, some of these antiphagocytic mechanisms will be highlighted below.

1.5.3.1  Modulation of Phosphorylation

One of the first steps in the initiation of Fc-mediated phagocytic signalling is phosphorylation of the tyrosine-based signalling domains of the receptor. This serves to dock secondary kinases and adaptor proteins to the site of phagocytosis and recruit other downstream mediators. Many of these adaptors and effectors themselves become phosphorylated. Indeed this usually is required for activation.

One of the earliest antiphagocytic effects of *Yersinia* was found to be at least partly dependent upon the virulence plasmid-expressed protein Yop2b, now called YopH (Bolin *et al*. 1988). If exogenously added to cell lysates, a massive dephosphorylation of host proteins was noticed, suggesting a role for YopH in down regulating phosphorylation-based signals (Fallman *et al*. 2002). Specific targets of this enzyme have not been fully elucidated; however several groups suggest the adaptor: Fyn Binding Protein (FYB), the actin-binding protein paxillin, Focal Adhesion Kinase (FAK) and p130 Cas (Fallman & Gustavsson 2005, Yuan *et al*. 2005). These and many other proteins rely upon phosphorylation for activation and the initiation of phagocytosis.
By modulating these and other signalling proteins, downstream actin polymerisation can be, if not controlled then at least disrupted to the point of phagocytic inhibition. Interestingly this has been shown to be a less localised effect than previously thought, as phagocytic inhibition by *Yersinia* can actually prevent the uptake of other phagocytic prey by the same cell (Fallman *et al.* 1995). Furthermore, targetting phosphorylated proteins to inhibit phagocytosis seems to be a common theme, as certain enteropathogenic *Escherichia coli* effectors appear to induce dephosphorylation of important host cell signalling proteins despite having no intrinsic phosphatase activity (Goosney *et al.* 1999).

1.5.3.2 Modulation of GTPases

Phosphorylation of key signalling components initiates the downstream signalling leading to formation of the phagocytic cup. Many bacterial effectors target, not the step that initiates signalling as above, but that which initiates actin polymerisation itself. Again, using *Yersinia* as a model, the following example highlights an effector that directly modulates host small GTPases.

Removal of the *Yersinia* virulence plasmid completely abrogates antiphagocytic effects (Rosqvist *et al.* 1988), however this is due to both YopH (see above) and a second major virulence factor YopE (Rosqvist *et al.* 1990). Further sequence analysis revealed that YopE contains a domain with homology to Rho-GAPs, that is capable of stimulating the intrinsic GTPase activity of Rho, Rac and Cdc42 (Von Pawel-Rammingen *et al.* 2000). Biasing GTPases to their GDP and thus inactive state, serves to focally inhibit the polymerisation of actin at the site of bacterial attachment. By deactivating the GTPases that are otherwise required for phagocytic internalisation, *Yersinia* can largely inhibit actin polymerisation and the subsequent formation of a phagocytic cup.

This behaviour is not limited to *Yersinia* species however, as another bacterium, *Pseudomonas aeruginosa* is also internalised and killed more efficiently in the absence of its virulence plasmid (Engel & Balachandran, 2009). Two proteins encoded by this plasmid, ExoS and ExoT, bear 95% amino acid sequence similarity and have been individually demonstrated to be important for virulence (Ernst *et al.* 2000). Both effectors bear an arginine finger domain, characteristic of RhoGAPs. Indeed, it has since been demonstrated that RhoGAP activity was largely responsible for its antiphagocytic capacity (Engel *et al.* 2000).
These examples serve to demonstrate how obligate extracellular bacteria can modulate the host cell signalling pathways. Other mechanisms of host modulation exist (Siemsen et al. 2009, Yeager et al. 2009); however, the bacteria exemplified here focus upon directly affecting phagocytic signalling. By targeting multiple, partially redundant steps that lead to the formation of a phagocytic cup, microorganisms can evade uptake and subsequent degradation. Furthermore, this antiphagocytic activity is essential for the bacteria to remain in an environment that suits their proliferation and survival.
1.6 Impetus

The work in this thesis identifies and addresses three topics of interest in the field. The basis for these choices is outlined below:

1.6.1 Project #1

As described in Section 1.5, microorganisms can modulate host cell signalling to prevent their uptake into internal compartments. For obligate extracellular microbial species such as *Yersinia* or *Pseudomonas* this is favourable, as internalisation surely leads to degradation. Many bacterial species however, cannot efficiently survive in the extracellular space and require internalisation into the host cell. These obligate intracellular microbes can exist either contained in membrane compartments or free in the cytosol, each approach having benefits and limitations.

One intracellular pathogen that can survive within an intracellular membrane compartment is *Salmonella enterica* serovar Typhimurium. This bacterium is closely related to serovar Typhi, the microorganism responsible for typhoid fever in humans. Through the use of a Type III Secretion System (T3SS) *Salmonella* Typhimurium can induce its uptake into phagocytic and non-phagocytic cells. This is achieved through a type of forced phagocytosis, whereby effectors modulate the actin cytoskeleton and induce uptake through a mechanism not unlike macropinocytic ruffling (Terebiznik *et al.* 2002). Many of the 15 identified *Salmonella* Type III effectors have been studied in the context of bacterial invasion, however few have been studied alone, in a cell-based system.

The first project aims to study an important effector of *Salmonella* virulence in isolation. Using an isolated system of epithelial cells and plasmid DNA expression, functions can be identified without the potentially antagonistic and confounding roles of other Type III effectors.

1.6.2 Project #2

In most cases, even in the presence of antiphagocytic effectors, microorganisms are successfully internalised and degraded by professional phagocytes. Fc-gamma receptors are one of the biggest players in this aspect of innate immunity. Using different techniques, much is known about the signalling pathways leading to Fc-mediated phagocytosis. Biochemically, the receptor-opsonin interactions have been well studied. Using pharmacological inhibition at different steps of the
pathway has helped to elucidate key players in the signalling pathway. Surprisingly though, little is known about the initiating events of phagocytosis.

Clustering of the receptors is thought to be required for the initiation of signalling. Mechanistically this is thought to lead to the formation of lipid ‘rafts’ and protein complexes that alter the diffusion of the Src-family kinases. With slowed diffusion, these kinases have more opportunity to interact with the immobilised receptors and initiate signalling.

In order to test this hypothesis, the second project aims to study changes in protein and lipid diffusion at the phagocytic cup before and during the initiation of signalling. This will be studied in the context of the lipid-modified Src-family kinases due to their role in the initiation of downstream phagocytic signalling.

1.6.3 Project #3

As has been emphasised previously, a pivotal part of phagocytic internalisation is the polymerisation of actin to form and extend a phagocytic cup with which to engulf targets. The majority of studies have looked at initiating events such as receptor clustering or ITAM-based signalling. An important consideration is that phagocytosis and formation of the phagocytic cup is a transient event. After a cell has completely internalised a particle, the existence of the particle inside the cell is arguably the only sign that phagocytosis has occurred. There is no ‘residue’ of the phagocytic cup, which begs the question of how does the signalling accompanying phagocytosis act to ‘reset’ the cell to the status quo ante and indeed is this even a requirement?

During phagocytosis, the polymerised actin at the base of the phagocytic cup may have to be depolymerised even during the event. Reasons for this include the regulated delivery of internal membranes to the forming phagosome and disassembly of a physical barrier to internalisation. Several studies have already tried to elucidate the mechanism by which GTPase activity is shut off after formation of the phagocytic cup (Lerm et al. 2007, Scott et al. 2005). These studies however, only cover small aspects of phagocytic signalling and as such, many of the regulatory mechanisms are yet to be determined.

The third section of this thesis will aim to determine initially, whether down regulation of actin at the phagocytic cup is required for completion of internalisation. Secondly, at what point in the
signalling pathway is there abrogation of signalling, to allow disassembly of the actin cytoskeleton.
1.7 References


Mao, Y.S., H.L. Yin. 2007. Regulation of the actin cytoskeleton by phosphatidylinositol 4-phosphate 5 kinases. Pflugers Arch. 455(1):5-18.


Chapter 2

2 Alteration of epithelial structure and function associated with phosphatidylinositol 4,5-bisphosphate degradation by a bacterial phosphatase.

2.1 Abstract

Elucidation of the role of PtdIns(4,5)P$_2$ in epithelial function has been hampered by the inability to selectively manipulate the cellular content of this phosphoinositide. Here we report that SigD, a phosphatase derived from Salmonella, can effectively hydrolyse PtdIns(4,5)P$_2$, generating PtdIns(5)P. When expressed by microinjecting cDNA into epithelial cells forming confluent monolayers, wild-type SigD induced striking morphological and functional changes that were not mimicked by a phosphatase-deficient SigD mutant (C462S). Depletion of PtdIns(4,5)P$_2$ in intact SigD-injected cells was verified by detachment from the membrane of the pleckstrin homology domain of phospholipase C-delta, used as a probe for the phosphoinositide by conjugation to green fluorescent protein. Single-cell measurements of cytosolic pH indicated that the Na$^+$/H$^+$ exchange activity of epithelia was markedly inhibited by depletion of PtdIns(4,5)P$_2$. Similarly, anion permeability, measured using two different halide-sensitive probes, was depressed in cells expressing SigD. Depletion of PtdIns(4,5)P$_2$ was associated with marked alterations in the actin cytoskeleton and its association with the plasma membrane. The junctional complexes surrounding the injected cells gradually opened and the PtdIns(4,5)P$_2$-depleted cells eventually detached from the monolayer, which underwent rapid restitution. Similar observations were made in intestinal and renal epithelial cultures. In addition to its effects on phosphoinositides, SigD has been shown to convert inositol 1,3,4,5,6-pentakisphosphate (IP$_5$) into inositol 1,4,5,6-tetrakisphosphate (IP$_4$), and the latter has been postulated to mediate the diarrhea caused by Salmonella. However, the effects of SigD on epithelial cells were not mimicked by microinjection of IP$_4$. In contrast, the cytoskeletal and ion transport effects were replicated by hydrolyzing PtdIns(4,5)P$_2$ with a membrane-targeted 5-phosphatase or by occluding the inositide using high-avidity tandem PH domain constructs. We therefore suggest that opening of the tight junctions and inhibition of Na$^+$/H$^+$ exchange caused by PtdIns(4,5)P$_2$ hydrolysis combine to account, at least in part, for the fluid loss observed during Salmonella-induced diarrhea.
2.2 Introduction

Phosphatidylinositol 4,5-bisphosphate (PtdIns(4,5)P$_2$) has long been recognised as an important source of second messengers. Hydrolysis of PtdIns(4,5)P$_2$ by phospholipase C yields diacylglycerol, a potent activator of most protein kinase C isoforms and other enzymes bearing C1 domains, and inositol 1,4,5-trisphosphate, which induces release of calcium stored in the endoplasmic reticulum (Taylor, 2002). In addition, phosphorylation of PtdIns(4,5)P$_2$ by class I phosphatidylinositol 3-kinases generates phosphatidylinositol 3,4,5-trisphosphate, a ligand and activator of various effectors that contain pleckstrin homology (PH) domains (Vanhaesebroeck et al., 2001; Lemmon, 2003). Not only are its metabolites critical for signal transduction, but PtdIns(4,5)P$_2$ itself serves multiple regulatory functions in the cell.

It affects several stages of actin microfilament assembly and remodeling, including uncapping of barbed ends, severing and bundling of filaments, and de novo nucleation (Hilpela et al., 2004; Roth, 2004). In addition, several studies have shown that a variety of ion channels and exchangers are directly modulated by the local concentration of PtdIns(4,5)P$_2$ (Leung et al., 2000; Hilgemann et al., 2001; Hilgemann, 2003). The functional importance of the metabolites generated from PtdIns(4,5)P$_2$ has been convincingly established by pharmacological means. Potent and reasonably specific phospholipase C and protein kinase C inhibitors are available, which have been used to evaluate the physiological role of diacylglycerol, inositol 1,4,5-trisphosphate (IP$_3$), and their effectors (Botelho et al., 2000; Matsui et al., 2001; Spitaler and Cantrell, 2004). Similarly, class I phosphatidylinositol 3-kinases can be selectively inhibited by wortmannin or LY294002 to assess the effects of phosphatidylinositol 3,4,5-trisphosphate biosynthesis (Vieira et al., 2001; Djordjevic and Driscoll, 2002). By contrast, establishing the function of PtdIns(4,5)P$_2$ has proven to be considerably more difficult. No specific inhibitors of the kinases that generate this phosphoinositide have been described, and the coexistence of multiple kinase isoforms and splice variants has precluded genetic analysis. Definitive confirmation of the involvement of PtdIns(4,5)P$_2$ in the regulation of ion channels and transporters stems largely from electrophysiological studies in excised patches or perfused cells, where the cytosolic aspect of the membrane can be accessed directly by solutions containing varying amounts of PtdIns(4,5)P$_2$ or bacterial lipases (Estacion et al., 2001; Loussouarn et al., 2003; Oliver et al., 2004). The multiple actions of PtdIns(4,5)P$_2$ in cytoskeletal dynamics have been similarly gleaned primarily from studies of disrupted cells (Nebl et al., 2000; Hsin-Yi et al.,
2004). Remarkably little is known about the role of PtdIns \((4,5)P_2\) in epithelial structure and function. The barrier and vectorial transport functions of epithelia are eminently dependent on the maintenance of the integrity of individual cells and of their intercellular contacts. This requirement rules out the use of most of the techniques that have been successfully applied to study PtdIns\((4,5)P_2\) function in other systems. Because molecular or genetic manipulation of the kinases that generate PtdIns\((4,5)P_2\) is subject to the limitations described above, we considered instead the possibility of modulating the cellular PtdIns\((4,5)P_2\) content by expression of phosphoinositide-specific phosphatases. Some success has been reported using Inp54p, a yeast inositol polyphosphate 5'-phosphatase (Raucher et al., 2000). In our hands, however, this enzyme, as well as the native forms of the mammalian phosphoinositide phosphatases synaptojanin, SKIP and OCRL, had negligible effects on the PtdIns\((4,5)P_2\) content of epithelial cells (unpublished data). Failure of the phosphatases to target to the plasmalemma and/or to become activated likely account for these observations. We reported that SigD/SopB, an injected virulence factor of *Salmonella* species, altered the binding of a PtdIns\((4,5)P_2\)-specific PH domain to the inner leaflet of the plasma membrane in HeLa cells (Terebiznik et al., 2002). We now present evidence that SigD/SopB (referred to hereafter as SigD) functions as a 4'-phosphatase that dephosphorylates PtdIns\((4,5)P_2\) to form PtdIns\((5)P\). By cloning this bacterial phosphatase into a mammalian expression vector we were able to introduce it by microinjection into intact epithelia, which are notoriously refractory to transfection. This strategy enabled us to analyse the consequences of selective depletion of PtdIns\((4,5)P_2\) in confluent epithelia. Because *Salmonella* is an enteric pathogen that injects SigD along with several other products into host epithelial cells via a type III secretion system encoded by the *Salmonella* pathogenicity island (SPI)-I (Galan, 1998), we focused our study primarily on IEC-18 cells, a line derived from the rat small intestine (Ma et al., 1992). In this manner, we simultaneously learned about the possible consequences of *Salmonella* infection on intestinal physiology.

### 2.3 Results

**SigD Hydrolyses Phosphatidylinositol 4,5-bisphosphate** : SigD contains a domain with homology to mammalian inositol 4-phosphatases (Norris et al., 1998). We reported earlier that SigD induces the displacement of PLCδ-PH-GFP, a ligand of PtdIns\((4,5)P_2\), from the membrane of HeLa cells (Terebiznik et al. 2002) and interpreted these results to mean that the bacterial protein modified PtdIns\((4,5)P_2\) in a manner that rendered it unable to associate with the PH
domain. However, SigD was also reported to act as an inositol polyphosphate phosphatase, capable of depleting cellular IP₆ and inositol pyrophosphates and of converting IP₅ into IP₄ (Norris et al., 1998). The latter can in turn be converted to IP₃, which interacts with high affinity with PH domains. It was thus conceivable that the displacement of PLCδ-PH-GFP from the membrane occurred as a result of IP₃ formation, without alteration in PtdIns(4,5)P₂. To determine whether SigD displays catalytic activity toward phosphoinositides in vivo we analysed the lipid composition of HeLa cells exposed to the phosphatase using HPLC. Phosphoinositides were labelled using [³H]-myoinositol and the cells were otherwise untreated (control) or were infected with Salmonella enterica serovar Typhimurium (S. Typhimurium) to obtain extensive and nearly synchronous delivery of bacterial proteins to the host cell cytosol, via their type-III secretion system. Cells were infected with either wild type or with sigD-deficient S. Typhimurium (ΔsigD), in order to assess the contribution of the phosphatase. The inositide content data were normalised to the amount of PtdIns, the predominant species in mammalian cells, which is thought to be practically invariant. As shown in Table 1, the PtdIns(4,5)P₂ content of control cells was equivalent to 8.2% of the PtdIns, in the range reported for other cells (Serunian et al., 1991). Infection for only 15 min with bacteria that express SigD (wild type) resulted in a 34% drop in the PtdIns(4,5)P₂ content, to 5.3% of the PtdIns. The decrease was consistently observed in three independent experiments and was statistically significant (P < 0.05 using ANOVA-Bonferroni’s multiple comparison test). The large decrease in PtdIns(4,5)P₂ was absent when infection was performed using SigD-deficient bacteria (Table 1). In this case, the drop was considerably smaller and not significant (P > 0.05). The preceding data are consistent with the notion that SigD actively dephosphorylates PtdIns(4,5)P₂ to PtdInsP. This was confirmed by analyzing the PtdInsP content of the samples (Table 1). In untreated cells the major PtdInsP peak detected by HPLC, which is comprised of PtdIns(4)P plus PtdIns(5)P, constituted 5.5% of the PtdIns. Infection with bacteria expressing SigD resulted in a considerable increase in PtdInsP, to 7.4% of PtdIns. Notice that the magnitude of the increase in PtdInsP is similar to the decrease in PtdIns(4,5)P₂ recorded under the same conditions, suggesting a precursor and product relationship. Importantly, the PtdInsP content was not increased when the cells were infected with SigD-deficient bacteria (Table 1), confirming that the phosphatase is responsible for the generation of the inositide.
Table 1: Analysis of Phosphoinositide Content After Salmonella Infection

Phosphoinositide levels were quantified before (Uninfected) and after infection of HeLa cells with either wild-type or sigD-deficient (delta-sigD) *Salmonella*. Lipids were extracted and analysed by HPLC as detailed in Materials and methods. The amount of the phosphoinositides is given as percent of PtdIns in the same sample. PtdInsP refers to the sum of both PtdIns(4)P and PtdIns(5)P, which are not resolved by the chromatographic system used. Data are means ± SEM of three separate experiments.
<table>
<thead>
<tr>
<th></th>
<th>Uninfected</th>
<th>Salmonella Wild Type</th>
<th>Salmonella ΔsigD</th>
</tr>
</thead>
<tbody>
<tr>
<td>PtdIns(4,5)P$_2$</td>
<td>8.2 ± 0.7%</td>
<td>5.3 ± 0.4%</td>
<td>6.9 ± 0.4%</td>
</tr>
<tr>
<td>PtdInsP</td>
<td>5.5 ± 0.7%</td>
<td>7.4 ± 0.9%</td>
<td>3.5 ± 0.2%</td>
</tr>
</tbody>
</table>
**SigD Generates Phosphatidylinositol 5-Phosphate:** Because SigD has homology to mammalian inositol 4-phosphatases (Norris *et al.* 1998), the disappearance of PtdIns(4,5)P₂ is likely the result of its conversion to phosphatidylinositol 5-phosphate (PtdIns(5)P). While the preceding data confirmed the near stoichiometric disappearance of PtdIns(4,5)P₂ and concomitant appearance of PtdInsP, the HPLC system used is unable to differentiate PtdIns(5)P from phosphatidylinositol 4-phosphate (PtdIns(4)P). To test whether PtdIns(5)P is in fact formed we used a combined enzymatic and TLC assay that involves conversion of PtdIns(5)P to PtdIns(4,5)P₂ by PtdIns(5)P 4-kinase. The identity of the product of the enzymatic reaction was validated by HPLC (for details see Materials and methods and Morris *et al.* (2000) and Niebuhr *et al.* (2002)). As above, to discern the contribution of SigD we compared the effects of wild-type *Salmonella* to those of mutants devoid of SigD. The deletion of sigD was verified by immunoblotting (Figure 2.1). As shown in Figure 2.1A, infection with wild-type, but not SigD-deficient *Salmonella*, generated a sizable amount of PtdIns(5)P, indicated by the formation of PtdIns(4,5)P₂. That this difference between the two bacterial strains was due to the deletion of sigD was confirmed by reintroduction of the phosphatase into the deficient *Salmonella*. Transformation of (ΔsigD) *Salmonella* with a plasmid encoding wild-type SigD restored the production of PtdIns(5)P upon infection (Figure 2.1A). Like other active phosphatases, SigD has an essential cysteine in its active site. We generated a plasmid encoding a mutant SigD where the critical cysteine was replaced by serine, namely SigD (C462S). SigD-deficient bacteria were then transformed with this plasmid and used to infect mammalian cells. Immunoblotting was used to confirm that the level of expression of the mutant and wild-type forms of SigD was similar in the strains used (Figure 2.1B). Unlike the plasmid encoding wild-type SigD, the C462S mutant plasmid was unable to restore the appearance of PtdIns(5)P (Figure 2.1C), confirming that the 4-phosphatase activity of the enzyme is responsible, at least in part, for the hydrolysis of PtdIns(4,5)P₂. In principle, PtdIns(5)P could have also been generated by dephosphorylation of PtdIns(3,5)P₂ on position 3, as has been reported for myotubularins (Tronchere *et al.*, 2004), or by sequential dual dephosphorylation of PtdIns(3,4,5)P₃, a preferred substrate of SigD *in vitro* (Norris *et al.*, 1998). Several lines of evidence argue against these possibilities. First, analysis by HPLC revealed that the cellular content of PtdIns(3,5)P₂ is far too low to account for the increase in PtdInsP. As shown in Figure 2.9A, the basal level of PtdIns(3,5)P₂ is equivalent to ~0.1% of...
Figure 2.1: Generation of PtdIns(5)P by SigD.

HeLa cells were infected with the indicated strains of *Salmonella* and, after 10 min at 37°C, the reaction was stopped and lipids were extracted as described in Materials and methods. The PtdIns(5)P content was next analysed by its enzymatic conversion to radiolabelled PtdIns(4,5)P$_2$ by insertion of the gamma phosphate of [$^{32}$P]ATP, catalysed by the type II phosphoinositide kinase, which selectively phosphorylates the 4’-position of PtdIns(5)P. The resulting lipids were next separated by thin layer chromatography. A representative chromatogram is shown in A. The site where the lipid mixture was spotted (Origin) and the position of the PtdIns(4,5)P$_2$ produced are indicated. The cells were infected, from left to right, with wild-type *Salmonella*, SigD-deficient *Salmonella*, SigD-deficient *Salmonella* bearing a plasmid expressing wild-type SigD cDNA, and SigD-deficient *Salmonella* bearing a plasmid expressing the catalytically inactive SigD mutant SigD (C462S). Lipids from uninfected cells are shown in the last lane. The deletion of SigD and the level of expression of the retransformed wild-type and mutant SigD were verified by immunoblotting. Identical amounts of bacteria were loaded, and lanes from the same gel and exposure are shown in B, which is representative of two similar blots. Lanes were separated in the image to facilitate alignment with the TLC (above) and bar graph (below). Molecular mass markers (in kD) are shown to the right. In C the amount of PtdIns(4,5)P$_2$ present in each TLC sample was quantified by HPLC and online continuous-flow liquid-scintillation counting. The chromatogram and quantitation shown are representative of three similar independent experiments.
the PtdIns, over 25 times lower than the increase in PtdInsP recorded in the same experiments. It is unlikely that PtdIns(3,5)P₂ is rapidly generated by other processes and simultaneously degraded by SigD, thus failing to accumulate. This is indicated by the finding that the PtdIns(3,5)P₂ content of cells infected with SigD-deficient bacteria is also extremely low, similar to that of untreated or wild-type *Salmonella*-infected cells (Figure 2.9A). Like PtdIns(3,5)P₂, the content of PtdIns(3,4,5)P₃ in resting cells is much too low (~0.1% of PtdIns) to account for the formation of PtdInsP, did not decrease upon infection with wild-type bacteria, and did not increase in cells infected with (ΔsigD) bacteria (unpublished data).

Additional evidence against the involvement of PtdIns(3,5)P₂ or PtdIns(3,4,5)P₃ in the generation of PtdIns(5)P was obtained using LY294002. This compound effectively inhibits both class I and class III PtdIns 3 kinases, which are required for the formation of PtdIns(3,4,5)P₃ and of PtdIns(3)P, the precursor of PtdIns(3,5)P₂, respectively. Figure 2.9B shows that under the conditions used, LY294002 blocked PtdIns 3-kinase activity; endosomal PtdIns(3)P, detected using a tandem FYVE domain from EEA1, disappeared upon treatment with the drug (panels B and B'). When cells were first treated with LY294002 and then infected with wild-type *Salmonella* in the presence of the inhibitor, the generation of PtdIns(5)P persisted (Figure 2.9C). Jointly, these observations provide convincing evidence that SigD from *Salmonella* is an effective phosphoinositide phosphatase capable of converting PtdIns(4,5)P₂ to PtdIns(5)P.

Whether the increase in PtdInsP measured by HPLC can be accounted for in its entirety by PtdIns(5)P remains to be defined, since the combined enzymatic/TLC assay does not yield quantitative estimates. Nevertheless, it is clear that at least a fraction of the PtdInsP generated is PtdIns(5)P.

**Effect of SigD on the PtdIns(4,5)P₂ Content of Epithelial Cells:** The IEC-18 cell line was used as an epithelial model for these studies for two reasons. First, it forms well-defined polarised monolayers with distinct junctional complexes (see below). Second, IEC-18 cells were derived from the small intestine, where *Salmonella* infection occurs in humans. Thus, our studies could potentially yield both basic and pathophysiological information. Confluent epithelial monolayers are notoriously difficult to transfect, and this was found to be the case for IEC-18 cells as well (unpublished data). To introduce cDNA into these cells without disrupting the integrity of the
Figure 2.2: Effect of SigD on PtdIns(4,5)P_2 distribution and cellular morphology.

Intestinal epithelial cells (IEC-18) were microinjected with cDNA encoding PLCδ-PH-GFP only (A-C), or together with either wild-type SigD (D-F) or SigD (C462S) cDNA (G-I). Cells were then incubated for 3 h at 37°C before imaging by confocal (A, D, and G), differential interference contrast (DIC; B, E, and H), or conventional epifluorescence microscopy (C, F and I). A, D, and G illustrate representative x vs. y confocal slices acquired near the middle of the cell, while A’, D’, and G’ are the corresponding x vs. z reconstructions. The DIC images in B, E, and H correspond to the cells in C, F, and I, respectively. The images are representative of at least 10 similar experiments of each type. Bar, 10 μm.
SigD as a phosphoinositide phosphatase when introduced into mammalian cells and highlight its ability to deplete PtdIns(4,5)P$_2$ in epithelial cells.

**Effect of SigD on Epithelial Morphology and Integrity:** In addition to the vacuolation reported in Figure 2.2, other structural changes were consistently noted in cells expressing SigD. These changes developed gradually over time and were always preceded by displacement of PLCδ-PH-GFP from the membrane, suggesting that they were a consequence of the hydrolysis of PtdIns(4,5)P$_2$. The development of the structural changes is illustrated in Figure 2.3. Concomitantly with the appearance of vacuoles, cells injected with SigD extend lamellipodia beyond the original junctional complexes (see Figure 2.3, B and B', and Figure 2.4 below). At later times blebbing of the apical surface is apparent (Figure 2.3, C and C'). These blebs extend well beyond the surface of the monolayer and are in fact best detected when the focal plane of the microscope is raised by 10 μm above the normal apical surface (Figure 2.3, H and H'). Eventually, most of the PtdIns(4,5)P$_2$-depleted cell bulges above the monolayer, rounding up (Figure 2.3, D-I) and finally detaching from the epithelium. All of these effects depend on the phosphatase activity of SigD, since they were never observed in cells expressing SigD(C462S). As shown in Figure 2.3(E and J), such cells retained normal morphology and remained as integral components of the monolayer even after 5 h, when cells expressing wild-type SigD had generally bulged and rounded up. Because PtdIns(4,5)P$_2$ effectively modulates the actin cytoskeleton, we speculated that the morphological changes induced by SigD were due, at least in part, to alterations in actin filament structure. To test this notion cells were fixed and permeabilised at various times after microinjection with cDNA encoding SigD and PLCδ-PH-GFP. The cells were then stained with rhodamine-phalloidin to reveal F-actin and analysed by confocal microscopy (Figure 2.4). These experiments indicated that actin underwent a biphasic change. At the early stages of action of SigD, the F-actin content of the cells seemingly increased, particularly in the lamellipodia that extended beyond the normal junctional boundaries (Figure 2.4, A and B). At this stage, the SigD-injected cells remained within the context of the monolayer, though the stress fibers attaching them to the substratum were somewhat depleted (not depicted). Subsequently, bulging above the monolayer and vacuolation became apparent (panels C and D, labelled ‘mid-stage’ in Figure 2.4). At this time the lamellipodia had receded and the net F-actin content of the cells had diminished. At even later stages the vacuoles were resorbed and the cells rounded up and protruded above the monolayer (Figure 2.4, E and F,
Figure 2.3: Time course of the effects of PtdIns(4,5)P₂ depletion on epithelial morphology.

Epithelial cells grown to confluence were used for microinjection of PLCδ-PH-GFP and SigD cDNA (1:5 ratio). Wild-type SigD was used in A-D and F-I, while SigD (C462S) was used in E and J. DIC and epifluorescence images were acquired with the focal plane near the apical surface of the cells (A-E) or ~10 μm above the apical membrane (F-J). The images shown are representative of at least five similar experiments. Bar, 10 um.
Figure 2.3
Figure 2.4: Effect of PtdIns(4,5)P2 depletion on F-actin and PtdIns(4,5)P2 distribution.

Intestinal epithelial cells (IEC-18) were microinjected with PLCδ-PH-GFP and SigD cDNA (1:5 ratio). The monolayers were incubated for varying periods of time and were then fixed, permeabilised, and stained with rhodamine-phalloidin to reveal F-actin. The stained cells were then analysed by confocal fluorescence microscopy. Panels A-F show PLCδ-PH-GFP in green and F-actin in red. Panels A'-F' show only F-actin in white. Panels A, A', C, C', E, and E' illustrate representative x vs. y confocal slices acquired near the middle of the cell, while B, B', D, D', F, and F' are the corresponding x vs. z reconstructions. A and B are representative of cells fixed at an early stage, i.e., 2-4 h after injection. C and D are representative of cells fixed at an intermediate stage, i.e., 4-5 h after injection. E and F are representative of cells fixed at a late stage, i.e., 5-6 h after injection. The images shown are representative of 10 similar experiments of each type. Bar 10 μm.
Figure 2.4
labelled ‘late stage’), ultimately detaching. Little F-actin remained at this stage, accounting for cell rounding. Of note, the neighboring cells rapidly occupied the space vacated by the PtdIns(4,5)P₂-depleted cell, a form of epithelial restitution.

The Effects of SigD on the Cytoskeleton Are Mediated by Depletion of PtdIns(4,5)P₂: As described above, the structural changes elicited by SigD were not mimicked by SigD(C462S). Because this inactive mutant also failed to alter PtdIns(4,5)P₂, depletion of the inositide is likely responsible for the observed structural changes. However, it is also possible that accumulation of PtdIns(5)P is involved and, since SigD hydrolyses several soluble inositol phosphates (Norris et al., 1998), generation of IP₄ from IP₃ is another potential cause of the morphological changes. The latter possibility was analysed by directly microinjecting the cells with IP₄. The concentration of IP₄ in the injection pipette was 100 μM. Because we estimate that the injection volume approximates 5-10% of the total cell volume, a final concentration of 5-10 μM IP₄ must have been delivered to the cells. This concentration is higher than the concentration of IP₄ reported in cells infected by Salmonella (Zhou et al., 2001). In four experiments, injection of IP₄ had no discernible effect on IEC-18 cell morphology or association with the monolayer (unpublished data). Similarly, microinjection of PtdIns(5)P or extracellular addition of this lipid in the presence of carriers that facilitate intracellular delivery of inositides was without effect on cell morphology (unpublished data). These observations suggest that depletion of PtdIns(4,5)P₂, not production of PtdIns(5)P or IP₄, was the cause of the morphological changes. To validate this conclusion, we manipulated PtdIns(4,5)P₂ by two independent procedures. First, we transfected a phosphoinositide phosphatase of mammalian origin, synaptojanin-2. To improve the efficiency of hydrolysis, a construct encoding the phosphatase domain was targeted to the membrane by addition of a prenylation motif, a polycationic sequence and CAAX box modeled after the C terminus of K-Ras. Expression of this construct effectively displaced PLCδ-PH-GFP from the membrane (Figure 2.5A; see Figure 2.2A for comparison with untreated control), indicating hydrolysis of PtdIns(4,5)P₂. More prolonged expression of the synaptojanin-2 phosphatase-CAAX construct induced cell blebbing (Figure 2.5B) and eventual detachment of the cells from the substratum. In parallel, the actin cytoskeleton was drastically altered, with marked disappearance of stress fibers (Figure 2.5C). We also tested the effects of over expression of an avid PtdIns(4,5)P₂ ligand, which at sufficiently high concentrations should mask a significant fraction of the inositide. To this end we used a construct of two tandem copies of
Figure 2.5: Alternative means of regulating PtdIns(4,5)P$_2$.

IEC-18 cells were microinjected with cDNA encoding for (A-C) the phosphatase domain of synaptojanin-2 fused to a CAAX box (PD-CAAX), or (D-F) two tandem copies of the PH domain from PLC$_\delta$-PH fused to GFP (see Materials and methods for details regarding use and construction). In A, the PD-CAAX DNA was coexpressed with the PLC$_\delta$-PH-GFP to identify transfected cells and to ensure the PtdIns(4,5)P$_2$ hydrolysis occurred. (A and D) Green fluorescence; (B and E) DIC images. The cell in D corresponds to E; (C and F) actin staining using phalloidin. Arrows indicate injected cells.
the PH domain of PLCδ, which is predicted to have enhanced avidity for PtdIns(4,5)P₂. As shown in Figure 2.5D, 2(PLCδ-PH)-GFP binds very effectively to the plasmalemmal PtdIns(4,5)P₂ and, when expressed at high levels, induced cell blebbing (Figure 2.5, D and E) and pronounced changes in cytoskeletal architecture (Figure 2.5F). Because the structural changes produced by synaptojanin-2 phosphatase-CAAX and 2(PLCδ-PH)-GFP resemble those induced by SigD, we believe that diminution in the amount of available plasmalemmal PtdIns(4,5)P₂ is the common underlying mechanism.

**Effect of PtdIns(4,5)P₂ Depletion on Tight Junction Integrity:** Since tight junctions are necessary to maintain epithelial integrity, which was lost upon expression of SigD, we suspected that depletion of PtdIns(4,5)P₂ may have destabilised the junctional complexes. This was tested by staining control and transfected monolayers with antibodies to ZO-1, a well-established tight junction marker. As reported earlier (Ma *et al.*, 1992), in confluent monolayers of IEC-18 cells ZO-1 was found to line the cellular junctions in a virtually continuous pattern (Figure 2.6A). Similar results were obtained in cells injected only with soluble or membrane-associated GFP constructs used as indicators of expression (not illustrated). Even at the early stages of the PtdIns(4,5)P₂ depletion process (1-3 h after injection) disruption of the junctional integrity was apparent, as judged by the discontinuities in the ZO-1-staining pattern (Figure 2.6, B and C). Preferential staining with ZO-1 at the cell boundary was completely eliminated at later stages. A disruption of ZO-1 architecture was also observed in cells expressing the synaptojanin-CAAX construct as well as in cells with high levels of 2(PLCδ-PH)-GFP expression. Importantly, the effect of SigD injection on junctional integrity was absent when the phosphatase-inactive mutant SigD(C462S) was used (unpublished data), pointing to depletion of PtdIns(4,5)P₂ as the underlying mechanism.

**Does SigD Induce Apoptosis of Epithelial Cells?:** The blebbing and rounding observed in cells expressing SigD is also characteristic of apoptotic cells (Majno and Joris, 1995). Moreover, infection with *Salmonella* has been reported to promote apoptosis of some cell types (Knodler and Finlay, 2001). It was therefore important to establish whether depletion of PtdIns(4,5)P₂ by SigD sufficed to trigger programmed cell death in IEC-18 cells. To this end, cells were coinjected with SigD or SigD(C462S) and a fluorescent protein, used as an injection and expression marker. Apoptosis was initially assessed from nuclear morphology in cells stained...
Figure 2.6: Effect of PtdIns(4,5)P2 depletion on junctional integrity.

Intestinal epithelial cells (IEC-18) were either untreated (A) or were microinjected with PM-GFP, a fluorescent microinjection and expression marker together with SigD cDNA (B and C). The monolayer was incubated for 2 h to allow expression and then fixed, permeabilised, and stained with antibodies to ZO-1, followed by Cy3-coupled secondary antibodies. The cells were analysed by fluorescence microscopy. Red fluorescence is shown in A and C and green in B. Arrows point to regions where junctional staining is discontinuous. Images are representative of five similar experiments. Bar, 10 µm.
with DAPI. A very small fraction of the uninjected cells (1-2%) had an apoptotic phenotype, consistent with findings in other cells (Majno and Joris, 1995). The sensitivity of the detection procedure was validated treating the cells with 100 nM of staurosporine for 2 or 5 h, which increased the apoptotic index to 13.4% and 34.9%, respectively. More importantly, when comparing over 100 cells from three experiments no significant increase in the fraction of apoptotic cells was detected in SigD-expressing cells (2%), even at the longest times tested. Longer times were not investigated, as the cells tended to detach from the monolayer (see Figure 2.3). DAPI staining is simple, yet not the most sensitive method for detection of apoptosis. Gross changes in nuclear morphology occur only in advanced stages of apoptosis and the time window of our experiments may have been insufficient to reach such stages. For this reason, we also assessed apoptosis using a more sensitive method that detects earlier stages of programmed cell death. Control and SigD-expressing cells were stained with a specific antibody that recognizes the activated form of caspase-3, an essential early component of the apoptotic chain. As in the case of DAPI, no significant difference in the fraction of apoptotic profiles was measured in four determinations between control and SigD-expressing cells (2% vs. 1%) while distinct apoptosis was observed following a short treatment with staurosporine (10.5% after 2 h). Jointly, these experiments indicate that apoptosis is not prevalent during the first 4-6 h of expression of SigD, at a time when PtdIns(4,5)P2 is extensively degraded and cell morphology is drastically altered. These results are consistent with the findings of Santos et al. (2001), who determined that SigD was not required for the induction of apoptosis in host cells in an animal model of Salmonella infection.

**Effect of PtdIns(4,5)P2 Depletion on Anion Permeability:** In the context of the intestinal epithelium, transient opening of the junctional complexes would be predicted to result in loss of vectorial ion transport and possibly cause diarrhea (Uzzau and Fasano, 2000). Indeed, SigD has been identified as a principal factor in causing diarrhea in animal models of Salmonella infection (Galyov et al., 1997). However, the loss of fluid was not attributed to loss of junctional integrity, but was instead proposed to be caused by increased Cl– secretion in response to elevated levels of IP4 (Norris et al., 1998). This conclusion, however, was derived from studies of chloride influx into nonepithelial (HEK293) cells and required validation in epithelia. We implemented measurements of anion permeability using a novel halide-responsive variant of the yellow fluorescent protein, YFP(H148Q). This protein responds to variations in halide concentration
Figure 2.7: Effect of PtdIns(4,5)P$_2$ depletion on anion permeability.

(A and B) IEC-18 cells were microinjected with YFP(H148Q), a halide-sensitive fluorescent protein with or without wild-type SigD cDNA. The cells were allowed to express the fluorescent probe for 2 h and subjected to digital imaging for assessment of halide permeability as described under Materials and methods. (A) Representative experiment. The emission of YFP(H148Q) is shown over time in a cell not expressing SigD. Where indicated, the concentration of chloride in the bathing medium was reduced by isoosmotic replacement with iodide. For calibration, the cells were permeabilised using tributyltin (TBT) and nigericin. (B) Quantitation of relative chloride permeability in cells expressing either YFP(H148Q) alone (control) or in combination with SigD. The rate of change of the fluorescence over time (delta F/t) was calculated following reduction of chloride to 0 mM by isoosmotic replacement with iodide. To allow comparison between experiments the fluorescence was normalised to the initial (maximal) value. Data are means ± SEM of 11 individual cells from three separate experiments. (C and D) Cells were microinjected with SigD cDNA as above or alternatively with the PD-CAAX construct and subsequently loaded hypotonically with MQAE as detailed in Materials and methods. Following a 30 min recovery period, the chloride concentration was manipulated using nitrate as a substitute and calibrated as in A. (D) Quantitation of the rate of change of MQAE fluorescence over time in control cells (open bar) with SigD (filled bar) or with PD-CAAX (gray bar). Data are means ± SEM of 121 cells from three separate experiments.
Figure 2.7
with changes in pKa that, at constant pH, translate into fluorescence changes (Jayaraman et al., 2000). IEC-18 cells were microinjected with the cDNA encoding YFP(H148Q), together with or without SigD. A typical measurement is illustrated in Figure 2.7A, where the concentration of Cl\(^-\) in the medium was varied stepwise, by iso-osmotic replacement with iodide. The latter anion is a more effective quencher of YFP(H148Q) emission, resulting in a progressive diminution of fluorescence. The loss was largely, but not entirely reversible, due to photobleaching incurred during repeated acquisitions. Indeed, a comparable partial decrease was noted upon repeated illumination at constant [Cl\(^-\)]. The bleaching component could be readily interpolated and corrected in our measurements. Finally, tributyltin and nigericin were used to calibrate the fluorescence vs. [Cl\(^-\)]. Using this approach, we were able to compare the rates of halide exchange in control cells and in cells expressing SigD. Cells were tested at the early and mid stages of expression, but not at the late stages (see Figure 2.3), because the tenuous attachment of the latter to the monolayer made measurements unreliable and calibration impossible. The data collected from 11 determinations are summarised in Figure 2.7B. Contrary to the predictions made on the basis of the work of Feng et al. (2001), we found that halide permeability was in fact depressed by SigD. Because GFP-derived probes such as YFP(H148Q) can be affected by environmental parameters other than the halide concentration, including the pH, it was imperative to ascertain that this unexpected discrepancy did not result from an experimental artifact. We therefore performed an independent set of experiments using a different chloride-sensitive probe, MQAE. While sensitive to halide concentrations, MQAE fluorescence is not altered by the physiological anions HCO\(_3\)^-, SO\(_4\)^-, and PO\(_4\)^-, by cations, or by pH (Verkman et al., 1989). Results of a typical experiment are shown in Figure 2.7C and the summary of 10 determinations from three separate experiments is presented in Figure 2.7D. As found using YFP(H148Q), the MQAE results indicate that anion permeability is diminished by treatment with SigD. To ensure that this diminution was caused by hydrolysis of PtdIns(4,5)P\(_2\), as opposed to dephosphorylation of inositol polyphosphates, we also tested the effects of PD-CAAX on chloride permeability. As shown in Figure 2.7D (gray bar), this membrane-associated, phosphoinositide-specific phosphatase produced an inhibition comparable to that seen with SigD, ruling out mediation by hydrolysis of soluble inositol polyphosphates. These findings are not consistent with elevated Cl\(^-\) secretion as the mechanism underlying SigD-induced diarrhea and, in addition, suggest a role for PtdIns(4,5)P\(_2\) in the modulation of epithelial anion transport.
Effect of PtdIns(4,5)P₂ Depletion on Na⁺/H⁺ Exchange: The apical membranes of intestinal and renal epithelial cells are active sites of Na⁺ resorption. Much of this absorption occurs in exchange for H⁺ and is coupled to Cl⁻/HCO₃ exchange and to osmotically obliged fluid absorption. There is evidence in model systems that some isoforms of the NHE are sensitive to the concentration of PtdIns(4,5)P₂ (Aharonovitz et al., 2000; Fuster et al., 2004), though this has not been validated for epithelial cells. The possible regulation of epithelial exchangers by PtdIns(4,5)P₂ is important not only in the context of Salmonella infection, but also because PtdIns(4,5)P₂ can vary during signaling, changes in cell volume, and ischemia. We therefore took advantage of the selective phosphatase activity of SigD and PD-CAAX to investigate the specific effects of PtdIns(4,5)P₂ depletion on Na⁺/H⁺ exchange activity in epithelial cells. Epithelial cells often coexpress multiple isoforms of NHE. To our knowledge, the isoforms present in IEC-18 cells have not been defined. Therefore, we first assessed the overall NHE activity of these cells and estimated the contribution of the ‘housekeeping’ NHE1 isoform and of specialised isoforms like NHE2 and NHE3. NHE activity was assessed as the Na⁺-induced recovery of the cytosolic pH from an acid load. Cytosolic acidification was imposed using an ammonium prepulse, as previously described (Aharonovitz et al., 2000). Preliminary experiments revealed that IEC-18 cells have a robust exchange activity (Figure 2.8A, a sample trace is shown in B, triangles). The contribution of individual isoforms to this response was investigated using HOE694, an inhibitor that differentially affects various NHE isoforms. At low (=1 µM) concentrations, HOE694 selectively blocks NHE1, while at higher concentrations (20 µM) it also blocks NHE2. Under these conditions, NHE3 is largely unaffected (Counillon et al., 1993). A different inhibitor has the converse effect; at 5 µM S3226 preferentially inhibits NHE3. As shown in Figure 2.8A, 82% of Na⁺/H⁺ exchange in IEC-18 cells was blocked by 1 µM HOE694. The fractional inhibition was not increased when 20 µM HOE694 was used, but an additional 11% was inhibited when 5 µM S3226 was added. These findings imply that Na⁺/H⁺ exchange in IEC-18 cells is largely mediated by NHE1, with a smaller contribution by NHE3. No NHE2 activity was detectable in these cells. We proceeded to assess the effect of PtdIns(4,5)P₂ depletion on exchange activity. As for Cl⁻ determinations, cells were tested at early and mid stages of expression, but not at the late stages, because the tenuous attachment of the latter to the monolayer made calibration impossible. Indeed, late stage cells become permeant to H⁺ equivalents and could not be acid loaded to the same degree as control cells. As illustrated in Figure 2.8 (B and C), depletion of PtdIns(4,5)P₂ by expression of SigD produced inhibition of
Figure 2.8: Effect of PtdIns(4,5)P2 depletion pH regulation.

To measure pH, IEC-18 cells were microinjected with YFP cDNA with or without wild-type SigD cDNA. The cells were allowed to express the proteins for 2 h. Alternatively, cells microinjected with PLCδ-PH-GFP with or without wild-type SigD or PD-CAAX cDNA were loaded with SNARF-5F during the final 30 min of the expression period. The cells were then subjected to digital imaging for assessment of cytosolic pH, as described under Materials and methods. (A) To define the functional contribution of individual NHE isoforms, the rate of pH recovery was measured in SNARF-5F loaded cells treated with the indicated inhibitors. From left: untreated cells; cells treated with 1 μM HOE694 (expected to inhibit NHE1 almost exclusively); cells treated with 20 μM HOE694 (expected to inhibit both NHE1 and NHE2); cells treated with 20 μM HOE694 plus 5 μM S3226 (expected to inhibit NHE1, NHE2 and NHE3). (B) Representative experiment showing pH recovery from an acid load in YFP (solid triangles) and YFP plus SigD-expressing cells (open circles). The emission of YFP was calibrated using nigericin and potassium, and the pH determined from such calibrations is shown over time. The cells were pre-pulsed with ammonium to induce cytosolic acidification upon its removal. Where indicated, sodium in the bathing medium was replaced isoosmotically by potassium and vice versa. (C) Quantitation of relative Na+/H+ exchange activity of control, SigD-expressing, or PD-CAAX-expressing cells. Activity was measured as the rate of sodium-induced pH recovery, measured over the first 1-2 min. Data show rates of alkalinisation and are means ± SEM of at least three experiments of each type.
Figure 2.8
NHE activity, which became more pronounced at the later stages of expression of this phosphatase. A significant inhibition of the antiport was also observed when cells were transfected with PD-CAAX, confirming that physiological PtdIns(4,5)P$_2$ levels are required for optimal NHE activity. These findings are in accord with the inositol sensitivity reported in nonepithelial model systems expressing NHE1 (Aharonovitz et al., 2000; Fuster et al., 2004) and NHE3 (Fuster et al., 2004).

2.4 Discussion

**SigD Is a PtdIns(4,5)P$_2$ Phosphatase:** The biological activity of SigD was first inferred from its sequence homology with mammalian phosphoinositol phosphatases (Norris et al., 1998). Its ability to dephosphorylate inositol phosphates was then demonstrated in vitro and cells expressing SigD were found to accumulate IP$_4$. It was subsequently appreciated, however, that SigD is largely membrane bound (Marcus et al., 2002), not an ideal location for the degradation of soluble substrates. Its subcellular location suggested that the degradation of inositol phosphates may not be the sole or even the most important function of SigD and prompted investigators to test whether it can additionally hydrolyse phosphoinositides. This notion was tested by in vitro experiments that were met with positive, yet conflicting, results; different groups confirmed that SigD is active against phospholipids, but the substrate selectivity varied among reports (Norris et al., 1998; Marcus et al., 2001; Hernandez et al., 2004). The first indication that SigD effectively cleaves phospholipids when introduced into mammalian cells was provided by Terebiznik et al. (2002), who described dissociation of PLC-δ-PH-GFP from the cytosolic face of the membrane in cells infected by wild-type, but not SigD-deficient, *Salmonella*. More recently, the appearance of PtdIns(3)P in *Salmonella*-containing vacuoles was also attributed to the lipid phosphatase activity of SigD (Hernandez et al., 2004). Here we provide direct biochemical evidence of phosphoinositide conversion induced by SigD. Cells infected by *Salmonella* were found to have a markedly elevated content of 4’ and/or 5’-phosphorylated PtdInsP, which corresponded quantitatively to the concomitant decrease in PtdIns(4,5)P$_2$. At least a fraction of the product was PtdIns(5)P, a lipid that is expressed in minute amounts in untreated cells. The increase in PtdIns(5)P induced by SigD is strongly reminiscent of the effect of IpgD, a *Shigella* virulence factor that bears considerable sequence homology (38.5%) with SigD. IpgD concomitantly produced depletion of PtdIns(4,5)P$_2$, pointing
to dephosphorylation of this inositide at the 4’ position as the source of PtdIns(5)P. Indeed, like SigD, IpgD bears a region of homology with mammalian inositol 4-phosphatases (Norris et al., 1998). Thus, both the Shigella and Salmonella phosphatases appear to target primarily PtdIns(4,5)P₂ and, as such, are very useful tools to study the functional requirement for this phosphoinositide in animal cells.

**Tools for the Manipulation of PtdIns(4,5)P₂ in Intact Mammalian Cells:** While phospholipids were initially thought to serve mainly a structural role, they are now believed to participate in signaling, vesicular traffic, and cytoskeleton regulation. Some of these suggested functions have been deduced from in vitro experiments but require verification in intact cells. Such verification has been most difficult, largely because selective manipulation of specific inositides has proven extremely complex. In the case of PtdIns(4,5)P₂, several strategies have been attempted. Metabolic inhibition has been found to be paralleled by depletion of PtdIns(4,5)P₂. However, the specificity of this manipulation is questionable, in view of the myriad ATP-dependent events in cells. PtdIns(4,5)P₂ is often depleted acutely by activation of phospholipase C using calcium ionophores (Varnai and Balla, 1998). As in the case of ATP depletion, however, the pleiotropic effects of calcium elevation make assignment of functional consequences ambiguous. More specific effects can, in principle, be obtained using phosphoinositide-specific phosphatases. Several 5-phosphatases have been identified and reported to cleave PtdIns(4,5)P₂. We have tested heterologous overexpression of OCRL and SKIP, with negligible effects on PtdIns(4,5)P₂ content (unpublished data). This may be attributed to the sub-cellular location of these enzymes, which are found mostly in endomembranes (Gurung et al., 2003; Ungewickell et al., 2004). In our hands, heterologous expression of Inp54p, a 5-phosphatase from yeast, had only minor effects on epithelial cells. We were similarly disappointed by the effects of overexpressed full-length synaptojanin (unpublished data), even though this phosphatase is found at the plasma membrane (McPherson et al., 1996). It is conceivable that synaptojanin, and possibly the other 5-phosphatases as well, require the coexpression of ancillary factors and/or are regulated by heretofore unidentified mechanisms. A constitutively active phosphatase that does not require cofactors and is not subject to regulation would be more suitable to acutely manipulate PtdIns(4,5)P₂. Our observations suggest that bacterial 4-phosphatases, such as SigD and IpgD, fulfill these requirements and have ample activity to induce massive degradation of PtdIns(4,5)P₂ in a variety of mammalian cells. These
enzymes can be expressed heterologously in eukaryotic cells by introduction of the available cDNAs, and recombinant proteins could in principle be used for micro-injection when even more acute effects are desired. Unlike the synaptojanin-CAAX construct, the bacterial phosphatases do not require posttranslational modifications, which facilitates recombinant expression and accelerates generation of the active protein in transfected cells. It must be borne in mind that both SigD and IpgD generate PtdIns(5)P in the plasmalemma as they degrade PtdIns(4,5)P2 and that this product may have biological activity, although none has been described to date and we failed to observe any effects following intracellular delivery of PtdIns(5)P. Nevertheless, a bioactive role for PtdIns(5)P cannot be ruled out.

**Effects of PtdIns(4,5)P2 Depletion on Epithelial Structure and Function:** Expression of SigD was accompanied by marked changes in the structure of epithelial cells. These were likely caused by the depletion of plasmalemmal PtdIns(4,5)P2, since neither PtdIns(5)P nor IP4, the known products of SigD activity, were able to recapitulate the structural changes. Evidence that PtdIns(4,5)P2 depletion can alter the actin cytoskeleton was provided by Raucher et al. (2000), who used optical tweezers to measure membrane tether forces in nonpolarised cells expressing Inp54p, a 5-phosphatase from yeast. Our results expressing synaptojanin-CAAX and 2(PLCδ-PH)-GFP are also consistent with a requirement for normal levels of PtdIns(4,5)P2 to maintain intact the epithelial structure. The effects of PtdIns(4,5)P2 depletion on cytoskeletal architecture followed a peculiar time course. At the earliest stages, the cells emitted apical lamellipodial extensions, and this coincided with extensive vacuolation. Subsequently, stress fibers disappeared gradually as the overall F-actin content diminished and the cells ultimately underwent blebbing and rounding as they protruded from the monolayer. Inactivation of ezrin likely contributed to disruption of the apical structure, since this protein is thought to anchor transmembrane proteins to actin fibers in a manner that is stimulated by PtdIns(4,5)P2 (Bretscher et al., 2002). The perijunctional actin band was also depleted upon hydrolysis of PtdIns(4,5)P2 and this was associated with loss of ZO-1 staining, consistent with junctional uncoupling. The loss of cell-cell contacts can readily explain the extrusion of the SigD-transfected cells from the context of the epithelial monolayer. To our knowledge, this is the first evidence that tight junctional integrity depends on the availability of PtdIns(4,5)P2. We also assessed the consequences of PtdIns(4,5)P2 depletion on a limited number of physiological parameters characteristic of epithelia. First, we noted that Na\(^+\)/H\(^+\) exchange was severely depressed by
reducing the level of the phosphoinositide. These observations are in good agreement with earlier reports in non-epithelial model systems (Aharonovitz et al., 2000; Fuster et al., 2004) and emphasize the multiplicity of actions of PtdIns(4,5)P2. It remains unclear whether the effects of the phospholipid are exerted directly or via intermediate proteins like ezrin (Baumgartner et al., 2004). In any event, it is clear that conditions predicted to alter PtdIns(4,5)P2, such as ischemia or infection by *Salmonella* or Shigella, will be accompanied by reduced salt and water absorption and may thus contribute to overall fluid loss during diarrhea (see below).

**Contribution of PtdIns(4,5)P2 Loss to Diarrhea Induced by Pathogenic Bacteria:** The occurrence of diarrhea is one of the major clinical complications of infection by *Salmonella*. Targeted deletion experiments initially demonstrated that the bacterial factors responsible for the fluid loss resided in the first pathogenicity island of the *Salmonella* genome (SPI 1). Further refinements of the deletional analysis indicated that the effector proteins SipA, SopA, SigD/SopB, SopD, and SopE2 act in concert to induce diarrhea (Zhang et al., 2002). The individual contribution of each of these proteins has not been thoroughly defined, but SigD is believed to be a major effector and has therefore been studied in most detail. In particular, it has been proposed that SigD contributes to fluid loss by stimulation of intestinal chloride secretion, and this effect was postulated to be mediated by IP4 (Feng et al., 2001). The rationale for this proposal is based on the earlier report that PtdIns(3,4,5)P3 acts as an inhibitor of calcium-dependent chloride secretion (Eckmann et al., 1997). By competing with PtdIns(3,4,5)P3, IP4 was proposed to relieve this inhibition, unmasking chloride secretion. In support of this hypothesis, Feng et al. (2001) reported that expression of SigD in HEK293 cells promoted an increase in chloride permeability. However, this hypothesis appears unlikely on the following grounds. (a) Chloride secretion is not constitutive and requires an increase in cytosolic calcium above the basal level. Indeed, the inhibitory effect of PtdIns(3,4,5)P3 is exerted on the secretion induced by muscarinic agents, but is not expected to occur in otherwise unstimulated cells. (b) The purported inhibitory effect of IP4 requires competition with existing PtdIns(3,4,5)P3. However, the resting levels of apical PtdIns(3,4,5)P3 in unstimulated cells are very low, insufficient to raise basal calcium by activation of phospholipase C-δ (Melendez et al., 1999) or of Tec-family kinases, if present (Carpenter, 2004). (c) Shigella, which is related to *Salmonella* and produces a similar diarrhea, was reported by Eckmann et al. (1997) not to generate IP4. (d) Lastly, the reported effects of SigD on chloride permeability appear not to be universal and, importantly, were not
observed in intestinal epithelial cells in our experiments. Jointly, these considerations detract from the weight that should be given to IP₄ and chloride secretion as contributing factors to *Salmonella*-induced diarrhea.

The experiments presented here suggest alternative mechanisms. First, depletion of PtdIns(4,5)P₂ by SigD resulted in inhibition of Na⁺/H⁺ exchange activity, which is predicted to depress the rate of sodium and water absorption (by inhibition of NHE3) and compromise intracellular pH homeostasis (by inhibition of NHE1). At later stages, cells expressing SigD lost F-actin and underwent opening of their junctional complexes. The ensuing transient increase in transepithelial conductance could readily account for decreased fluid absorption. Indeed, junctional opening has been invoked in the etiology of diarrhea in both bacterial and viral infections (Dickman et al., 2000; Goosney et al., 2000; Bertelsen et al., 2004). Clearly, though informative, microinjection of SigD into IEC-18 cells is not a perfect model of bacterial infection. This paradigm is useful in that it enabled us to isolate the contribution of SigD to the bacterial effector phenotype. On the other hand, neither the intensity nor the duration of the effects of expressed SigD are likely to be identical to those it exerts during bacterial infection. In the heterologous expression system SigD is likely to localize to both apical and basolateral membranes, which may not be the case during infection. Lastly, though derived from the rat ileum (Ma et al., 1992), IEC-18 cells are not perfect mimics of the primary epithelium. Nevertheless, the information obtained revealed the potential of SigD to exert a variety of biological effects, the contribution of which to the pathogenicity of the bacteria will need to be validated in vivo.

2.5 Materials and Methods

**Materials and Solutions:** IP₄ was purchased from Matreya Inc. Biochemicals. Rhodamine-phalloidin, 4′,6-diamidino-2-phenyl-indole (DAPI), FM4-64, SNARF-5F, N-(ethoxycarbonylmethyl)-6-methoxyquinolinium bromide (MQAE), and Alexa 568 microinjection dye were from Molecular Probes. Radiolabelled [³²P]ATP and [³H]-myoinositol were from MP Biomedicals (formerly ICN Biomedicals). The Na⁺/H⁺ exchanger (NHE) inhibitors HOE694 and S3226 were gifts of Hoechst and Aventis, respectively. FBS, a-modified Earle’s medium (MEM), Dulbecco’s modified Eagle’s medium (DMEM), HEPES-buffered solution RPMI-1640 (HPMI), PBS, and penicillin plus streptomycin were from Wisent.
Antibodies to ZO-1 were purchased from Zymed Laboratories. Monoclonal antibodies against active caspase 3 were from BD Biosciences. Cy3-conjugated secondary antibodies were from Jackson ImmunoResearch Laboratories. All other reagents were from Sigma-Aldrich.

Microinjection buffer consisted of (in mM) 140 KCl plus 10 HEPES, pH 7.4 at 37°C and was filter sterilised before use. Na⁺-rich solution contained 130 NaCl, 3 KCl, 1 CaCl₂, 1 MgCl₂, 20 HEPES, pH 7.4 at 37°C. In K⁺-rich solution, all NaCl was replaced by KCl, and in Cl⁻ depleted solution NaCl was replaced by NaI or NaNO₃.

**Cell Culture:** HeLa, IEC-18, and OK cells were obtained from the American Tissue Culture Collection. IEC-18 cells were grown in DMEM supplemented with 5% FBS and 0.1 U/ml bovine pancreatic insulin. HeLa cells were grown in DMEM supplemented with 10% FBS. OK cells were grown in a-MEM supplemented with 10% FBS. All cells were maintained under 5% CO₂ at 37°C. When required, cells were pretreated with 100 μM LY294002 for 30 min and the inhibitor was kept in the medium throughout the experiment.

**DNA Constructs:** The mammalian expression vectors encoding wild-type SigD and the inactive SigD mutant (C462S) have been described elsewhere (Marcus et al., 2001). The vector pEGFP::PLCδPH encodes the PH domain of PLCδ fused to eGFP (PLCδ-PH-GFP). pGFP::PM encodes the myristoylation/palmitoylation sequence from Lyn fused to GFP (PM-GFP). Both of these were the gift of T. Meyer (Stanford University, Stanford, CA) and their construction has been described before (Teruel et al., 1999). 2FYVE -GFP consists of two tandem FYVE domains from EEA1 conjugated to GFP and has been described previously (Vieira et al., 2001). The 2(PLCδ-PH)-GFP construct was made by combining two tandem PLCδ-PH domains to GFP and was a gift of M. Rebecchi (State University of New York, Sunnybrook, NY). The phosphatase domain of mammalian synaptojanin 2 fused to a CAAX box modeled after the carboxy terminal sequence of K-Ras (PD-CAAX) was the gift of M. Symons (The Feinstein Institute for Medical Research, Manhasset, NY). The construction of this construct has been detailed previously (Malecz et al., 2000). The plasmid encoding the anion-sensitive mutant of the yellow fluorescent protein YFP(H148Q) was described in Jayaraman et al. (2000).

**Bacterial Culture and Infection Protocol:** The source and properties of wild-type and SigD-deficient Salmonella enterica serovar Typhimurium, (delta-sigD) SL1344, were described previously (Terebiznik et al., 2002). For complementation analysis, the delta-sigD mutant of S.
Typhimurium SL1344 was transformed with plasmid pACYC184 encoding either wild-type or the catalytically inactive C462S mutant of SigD (Marcus et al., 2001). That the level of expression of the wild-type and mutant SigD was similar in the bacterial strains used was verified by immunoblotting, using a polyclonal anti-SigD antibody. Overnight bacterial cultures were diluted 1:30 into Luria-Bertani broth and incubated at 37°C, shaking for 3 h. Bacteria were sedimented at 10,000 g for 2 min and then resuspended in HPMI, pH 7.4. 1 ml of bacterial suspension was added to cells that had been plated on 10-cm dishes and preincubated with 1 ml of HPMI at 37°C for 5 min. Infection was performed at 37°C under 5% CO2. After 10 min, excess bacteria were washed away with PBS and the cells subjected to lipid extraction as detailed below.

**Lipid Extraction and Phosphoinositide Analysis:** Lipid labeling and extraction were performed essentially as described by Carricaburu et al. (2003). In brief, HeLa cells were labelled with 20 μCi/ml [3H]-myoinositol for 24-48 h in inositol-free medium. After labeling, the cells were washed free of excess isotope, infected with the indicated strain of *Salmonella* for 15 min, and immediately lysed in 1 M HCl. Lipids were next extracted in chloroform:methanol (1:1, vol:vol) and deacylated as described (Serunian et al., 1991). Deacylated lipids were separated by anion-exchange high performance liquid chromatography (HPLC), detected by an online Radiomatic detector (Perkin Elmer), and quantified relative to PtdIns using the ProFSA analysis program. Individual peaks in the chromatogram were identified using *in vitro*-synthesised internal standard lipids. For analysis of phosphatidylinositol 5-phosphate (PtdIns(5)P) the cells were treated as specified in the text and phospholipids were extracted in acidified chloroform:methanol as in Niebuhr et al. (2002). The lipid extracts were dried under nitrogen and used subsequently for determination of PtdIns(5)P content by the method of Morris et al. (2000). In brief, PtdIns(5)P was converted to PtdIns(4,5)P2 *in vitro* by addition of phosphatidylinositol 5-phosphate 4-kinase and 32P-ATP. The products of this reaction were separated by thin layer chromatography (TLC) and the amount of radiolabelled PtdIns(4,5)P2 quantified. The identity of the labelled product was confirmed to be PtdIns(4,5)P2 by analyzing the samples using HPLC.

**Microinjection Protocol:** Cells were grown on 25-mm glass coverslips and used for experiments 2 d after the monolayer had reached confluence. The coverslips were then transferred to a thermostatted Leiden chamber and incubated with HPMI medium supplemented
with antibiotic/antimicotic mixture (1:500) for 30 min before microinjection. The microinjection solution contained 50 μg/ml of the indicated mixture of plasmids, typically a 5 to 1 ratio of SigD to PLCδ-PH-GFP cDNA. Microinjection was performed under phase contrast microscopy using an Eppendorf Transjector 5246 controlled by an Eppendorf 5171 Micromanipulator. Microinjected cells were identified by the expression of the fluorescent protein products. To minimize evaporation during prolonged observation periods the chambers were sealed using a second coverslip secured with a small amount of silicon grease.

**Cytosolic pH Determinations:** For cytosolic pH determinations IEC-18 cells were microinjected with cDNA encoding YFP, with or without SigD cDNA. Cells were next incubated for 3 h at 37°C under 5% CO₂ to allow expression of the proteins. Alternatively, the cells were loaded with SNARF-5F by loading with 20 μM of the precursor acetoxymethyl ester for 30 min. The coverslips were then mounted in a thermostatted Leiden holder, bathed in a Na⁺-rich buffer, and placed on the stage of a Leica fluorescence microscope equipped with a PL Fluor 100x/1.30 N.A. oil immersion objective. Sutterfilter wheels positioned excitation and emission filters in front of a Hg lamp and the acquisition camera, respectively. For YFP, excitation was at 480 nm and was directed to the cells through a 510-nm dichroic mirror. Emitted fluorescence was selected through a 535BP25-nm filter. For SNARF-5F, excitation was at 550 nm and emission was recorded at 580 and 640 nm. Images were captured with an Orca ER cooled charge-coupled device camera (Hamamatsu). Image acquisition was controlled by the Meta fluor software v3.5 (Universal Imaging Corp.). To determine background, an area identical to the region of interest was selected outside the transfected cell and fluorescence was acquired. At the end of the experiment, a calibration curve of fluorescence (ratio) vs. pH was obtained *in situ* by sequential perfusion with K⁺-rich medium buffered to predetermined pH values (between 6.0 and 7.4) containing 10 μg/ml nigericin. Calibration curves were constructed by plotting the extracellular pH, assumed to be identical to the cytosolic pH under these conditions, against the corresponding fluorescence. To determine the relative contribution of specific NHE isoforms, pH determinations were performed in the presence of 1 μM HOE694 to selectively inhibit NHE1, 20 μM HOE694, to inhibit both NHE1 and NHE2, or 20 μM HOE694 plus 5 μM of S3226, to inhibit the three main plasmalemmal isoforms.

**Determinations of Anion Permeability:** Anion permeability was estimated by two methods: first, the anion-sensitive variant of YFP(H148Q) was used by a micro fluorimetric method
similar to that described above for pH determinations. Because YFP derivatives are also inherently sensitive to pH, a second series of experiments was performed using a chloride-sensitive dye, N-(ethoxycarbonylmethyl)-6-methoxyquinolinium bromide (MQAE), which was loaded into the epithelial cells by hypotonic stress, following expression of SigD or the PD-CAAX construct. To identify the cells expressing the phosphatase, SigD was cotransfected with PLCδ-PH-GFP. By studying the presence and distribution of this construct we not only identified the transfectants, but ensured that the phosphatase had in fact exerted its effects on PtdIns(4,5)P2. Control experiments were performed in cells expressing PLCδ-PH-GFP only. After loading hypotonically with MQAE, cells were allowed to recover in isotonic medium for 30 min before measurement of anion permeability. Calibration was made using 10 μM nigericin and 10 μM tributyltin chloride in K+-rich media of varying Cl− concentration.

Staining with Fluorescent Markers and Confocal Microscopy: Cells grown on coverslips were washed twice with PBS and fixed in 4% paraformaldehyde in PBS at room temperature for at least 30 min, followed by quenching of excess fixative with 100 mM glycine for 10 min. Cells were blocked while permeabilised using 5% skimmed milk in PBS containing 0.1% Triton X-100 for 60 min. ZO-1 was immunostained using a polyclonal primary antibody (1:100), followed by secondary Cy3-conjugated donkey anti-rabbit antibodies (1:1,000). To label F-actin, fixed and permeabilised cells were stained with a 1:500 dilution of rhodamine-phalloidin for 30 min. Two different methods were used for assessment of apoptosis. In both cases the cells were fixed with 3% paraformaldehyde for 15 min at room temperature and, where indicated, permeabilised with 0.1% saponin in PBS. For caspase-3 staining the preparation was blocked for 30 min in medium containing 10% donkey serum in PBS, and then incubated with antibody to active caspase-3 (1:200), followed by fluorescently conjugated secondary antibodies, each for 1 h. For DAPI staining, 5 μg/ml of the nuclear dye was added to the fixed and permeabilised cells, followed by incubation for 30 min at room temperature. Coverslips were mounted onto slides using DAKOô, left to dry overnight in the dark at room temperature, and then stored at -20°C. Both live and fixed samples were analysed by conventional epifluorescence microscopy using a Leica IRE DR2 inverted microscope with an Orca II ER camera (Hamamatsu) driven by the Openlab 3 software (Improvision) installed on an Apple G4 computer. Alternatively, analysis was made by confocal microscopy using a Zeiss LSM 510 laser scanning microscope with oil immersion objectives. Where indicated, the location of the plasma membrane was defined by addition of
FM4-64 (20 μM) at the time of imaging. FM4-64 is a solvochrome red fluorescent dye that partitions into the outer monolayer of the plasmalemma. GFP, Cy3 and FM4-64 were examined using the conventional laser excitation lines and filter sets.

2.6 References


2.7 Supplementary Figures

Figure 2.9: Phosphoinositide changes induced by SigD.

Lipids were extracted from HeLa cells after *Salmonella* invasion and analysed using HPLC. (A) Cells infected with wild-type (SWT) or SigD-deficient (delta-SigD) *Salmonella*. Black bar, change in PtdInsP content induced by SWT. Open bars, absolute content of PtdIns(3,5)P₂ before (control) and after infection. (B) IEC-18 cells expressing 2FYVE-GFP before (B) and after (B’) treatment with LY294002. (C) The PtdIns(5)P content analysed as in Fig. 1 from cells treated as indicated.
Figure 2.9

Panel A:
- Bar graph showing the percentage of PI.
- Δ[PI(4)P + PI(5)P] compared to PI(3,5)P_2.
- Controls and mutants (WT, ΔSigD).
- Δ=0.02% and Δ=0.00%.

Panel B:
- Images labeled -LY and +LY.
- Comparing cell morphology with and without LY treatment.

Panel C:
- Diagram showing PI(4,5)P_2 turnover.
- Control, WT, ΔSigD, WT+LY, +Salmonella conditions.
Figure 2.10: Effect of SigD in renal epithelial cells.

The expression of SigD was studied in opossum kidney (OK) cells. Morphological changes were studied alongside PtdIns(4,5)P₂ distribution as in Fig. 2.
Chapter 3

3 Signal-dependent immobilisation of acylated proteins in the inner monolayer of the plasma membrane.

3.1 Abstract

Phospholipids play a critical role in the recruitment and activation of several adaptors and effectors during phagocytosis. Changes in lipid metabolism during phagocytosis are restricted to the phagocytic cup, the area of the plasmalemma lining the target particle. It is unclear how specific lipids and lipid-associated molecules are prevented from diffusing away from the cup during the course of phagocytosis, a process that often requires several minutes. We studied the mobility of lipid-associated proteins at the phagocytic cup by measuring fluorescence recovery after photobleaching. Lipid-anchored (diacylated) fluorescent proteins were freely mobile in the unstimulated membrane, but their mobility was severely restricted at sites of phagocytosis. Only probes anchored to the inner monolayer displayed reduced mobility, whereas those attached to the outer monolayer were unaffected. The immobilisation persisted after depletion of plasmalemmal cholesterol, ruling out a role of conventional “rafts”. Corralling of the probes by the actin cytoskeleton was similarly discounted. Instead, the change in mobility required activation of tyrosine kinases. We suggest that signaling-dependent recruitment of adaptors and effectors with lipid binding domains generates an annulus of lipids with restricted mobility.

3.2 Introduction

Elimination of pathogens by phagocytosis is an essential component of the innate immune response. Phagocytosis is a complex sequence of signaling and cytoskeletal remodeling events that culminates in the engulfment of microorganisms into a vacuole. The process is initiated by recognition of ligands on the surface of the pathogen by specialised phagocytic receptors. Progressive zippering of receptors to multiple ligands on the target particle drives the apposition of the host cell membrane to the surface of the pathogen. In this specialised area of contact, known as the phagosomal cup, receptor clustering unleashes a signaling cascade that ultimately promotes actin polymerisation, pseudopod extension, and particle internalisation. Phosphoinositides play a critical role in the initiation of phagocytosis. Phosphatidylinositol-4,5-bisphosphate (PtdIns(4,5)P₂) undergoes a biphasic change at the phagocytic cup: an initial,
transient increase that is followed by its virtual disappearance by the time phagosomal sealing is complete (Botelho et al., 2000). These changes appear to be essential for successful completion of phagocytosis, as interference with PtdIns(4,5)P₂ biosynthesis or catabolism impairs particle engulfment (Azzoni et al., 1992; Liao et al., 1992; Araki et al., 1996; Cox et al., 1999). Conversion to phosphatidylinositol-3,4,5-trisphosphate (PtdIns(3,4,5)P₃) is partly responsible for the disappearance of PtdIns(4,5)P₂ from the phagosomal cup. Accordingly, formation of PtdIns(3,4,5)P₃ can be readily detected at the base of the nascent phagosome (Marshall et al., 2001), and inhibition of phosphatidylinositol-3-kinases, which are responsible for its synthesis, effectively blocks the uptake of phagocytic particles that are >3 μm (Cox et al., 1999).

Remarkably, the reported changes in PtdIns(4,5)P₂ and PtdIns(3,4,5)P₃ are confined to the phagocytic cup, without detectable alteration of the inositides in the unengaged (bulk) plasma membrane (PM). Biological membranes are generally regarded as fluid mosaics wherein lipids or clustered lipid microdomains can diffuse freely (Galbiati et al., 2001). At physiological temperatures, such unrestricted lateral diffusion would result in rapid redistribution and homogenisation of phospholipids. It is therefore unclear how gradients of inositides can be sustained for the time required for phagosome formation, which can exceed 3-4 min for large particles. Two possibilities can be envisaged: first, the lipids may be continuously generated at sites of phagocytosis and, although able to diffuse, they may be rapidly hydrolysed as they leave the cup. Thus, a dynamic steady-state gradient could be achieved. Alternatively, the diffusion of lipids at sites of phagocytosis may be restricted, differing from their mobility in the bulk of the plasmalemma. Mobility may be restricted within or across the boundary of the phagocytic cup. Lipids are important determinants of the distribution of membrane proteins. Extrinsic proteins can associate with lipid headgroups, and transmembrane proteins segregate into microdomains according to the nature of the surrounding lipids. Several important signal transduction proteins associate with membranes by inserting their acyl and prenyl moieties into the hydrophobic domain of the bilayer. It can be anticipated that changes in the lipidic composition of the membrane during phagocytosis would have important consequences on the distribution and hence the activity of signaling proteins. In fact, phosphoinositides are thought to contribute to signal transduction by recruiting adaptor and effector proteins to sites of phagocytosis (Stauffer and Meyer, 1997; Hinchliffe et al., 1998; Oancea et al., 1998; Varnai et al., 1999; Botelho et al., 2000). Clearly, the distribution and mobility of lipids and lipid-associated proteins is critical for vectorial transduction of signals during phagocytosis. However, the mobility of specific lipids in
native membranes is difficult to analyse. Introduction of fluorescent moieties can alter the size, charge, and/or conformation of their headgroup or tail, and defined labelled lipids are rapidly converted to other chemical species. An alternative method frequently used to study lipids in cells, namely, the expression of fluorescent chimeric proteins containing specific lipid binding domains, is of limited use to study mobility. The limitation stems from the fact that the complex formed between the lipid and the chimera is in rapid dynamic equilibrium, with dissociation occurring much faster than the movement of the lipid in the plane of the membrane (Marshall et al., 2001). Because of these limitations and because of their importance in signal transduction, we decided to analyse instead the mobility of lipid-linked proteins at the phagosomal cup. FRAP was used for this purpose. Various constructs were used that targeted either the inner or outer monolayer of the plasmalemma and that resided preferentially or, alternatively, were excluded from areas rich in saturated lipids. Using large phagocytic targets and a combination of bright-field and confocal fluorescence microscopy, we were able to establish that the mobility of saturated lipids is drastically reduced at the phagocytic cup by a process that requires receptor-induced tyrosine phosphorylation.

3.3 Results

**Mobility of PM-GFP in resting cells:** The mobility of membrane-associated molecules in activated macrophages was studied earlier by sedimentation of suspended cells onto IgG-coated surfaces (Marshall et al., 2001). This system has distinct optical advantages, as the membrane becomes activated at a fixed, predictable focal plane. However, this model of abortive phagocytosis does not recapitulate all aspects of the engulfment process and may involve components of cell spreading onto the substratum. On the other hand, phagocytosis of small particles is not amenable to the study of lipid mobility because of the rapidity of the internalisation event and the small cup size (Figure 3.8). As an alternative, we used large (8.3-μm diameter) particles as phagocytic targets. The size of these particles is similar to that of apoptotic cells that are commonly ingested by macrophages (Fadok et al., 1992). The distribution of PM-GFP in macrophages is shown in Figure 3.1. PM-GFP is a chimeric construct of the N-terminal 10 amino acids from Lyn with GFP. The N-terminal sequence of Lyn directs myristoylation and palmitoylation of the chimera, which targets the fluorescent protein to the inner monolayer of the plasmalemma (Teruel et al., 1999). Transverse (x vs. y; Figure 3.1A) and
Figure 3.1: Distribution and photobleaching of PM-GFP in macrophages.

RAW264.7 cells were transfected with PM-GFP and analysed by confocal laser-scanning microscopy. (A) Transverse (x vs. y) optical slice acquired near the middle of an otherwise untreated cell. Bar, 5 um. (B) Sagittal (x vs. z) reconstruction. (C) Transverse slice of the same cell after photobleaching of the area indicated by the circle. (D) Three-dimensional rendering of the photobleached cell. Note that only the front (bleached) half of the cell is illustrated, to facilitate visualisation of the bleached area. Bars, 2 um. Images in A–D are representative of >20 experiments. (E) Course of FRAP. An absolute intensity trace showing a typical bleach of ~90% is illustrated. (F) Normalised recovery after photobleaching. Shown are recovery of PM-GFP, BODIPY-labelled phosphatidylethanolamine (PE), and NBD-labelled phosphatidylserine (PS). Bleaching was performed at the arrow. Data are means ± SEM of eight individual experiments. Where absent, error bars were smaller than the symbol.
sagittal (x vs. z; Figure 3.1B) sections of the cells confirm that PM-GFP is largely plasmalemmal, although varying amounts of endomembrane staining can be seen, depending on the expression level. The mobility of PM-GFP was initially assessed by FRAP in unstimulated cells. As shown in Figure 3.1C and particularly in the three-dimensional reconstruction of Figure 3.1D, the optical setup used bleached a nearly circular area of ~2 μm in diameter within 1-2 s. Under the conditions of our experiments, ~10-20% of the original intensity remained after bleaching (Figure 3.1E). In otherwise untreated cells, the fluorescence recovered almost completely (Figure 3.1F, squares; and Table 2); in eight determinations, the mobile fraction (MF) averaged 1.10 ± 0.04 (these and all subsequent data are presented as means ± 1 SEM of the indicated number of determinations). Recovery was half maximal (t_{1/2}) after 15 ± 2 s, indicative of a diffusion coefficient of 1.5 x 10^{-10} cm²/s. This value is very similar to that we find for phospholipids in these cells. As shown in Figure 3.1F and Table 2), fluorescently labelled phosphatidylserine and phosphatidylethanolamine recover from photobleaching with comparable kinetics, yielding diffusion coefficients 2.4 x 10^{-10} and 2.2 x 10^{-10} cm²/s, respectively. Together, these findings imply that the mobility of PM-GFP in the membrane is limited by association of its acyl chains with other constituents of the bilayer and not by drag imposed by the GFP itself. Accordingly, the diffusion coefficient of free GFP in the cytosol has been estimated at 2.5 - 3.0 x 10^{-7} cm²/s (Swaminathan et al., 1996), much faster than that of the diacylated construct.

**Mobility of PM-GFP at the phagocytic cup:** RAW cells were exposed to IgG-opsonised latex beads to assess the mobility of PM-GFP at the phagocytic cup (Figure 3.2). The considerable time required for complete engulfment of the large beads (~6 min; Figure 3.8) enabled us to perform photobleaching and measure the recovery of fluorescence before phagocytosis was completed. Figure 3.2 (A-C) shows that the area of the membrane engaged in particle recognition and engulfment (the cup) was readily identifiable and sufficiently large to accommodate the ~2μm bleaching zone that was used. To normalize the recovery for changes in focal plane, photobleaching, or de novo delivery of probe, recovery was also measured at an area of unengaged membrane of the same cell. Although theoretically the MF cannot exceed 100%, membrane convolution at sites of ingestion can occasionally cause the MF to exceed this value. Typical results are shown in Figure 3.2D. Although the unengaged area of the membrane behaved as described for the membrane of resting cells (MF = 0.8-1), the fraction of mobile PM-
Table 2: Summary of Fluorescence Recovery After Photobleaching (FRAP) at the unengaged plasma membrane versus the phagosomal cup.

Cells were transfected or loaded with the labelled probe and analysed by confocal laser-scanning microscopy FRAP. Two areas of 2 μm in diameter were define, one at the phagosomal cup and a second one at an unengaged portion of the plasmalemma of the same cell. After acquiring three basal readings, the selected regions were irreversibly photobleached. The recovery of fluorescence was then monitored over time by scanning both areas. Data were analysed as described in Methods.
Table 2  Summary of FRAP results at the unengaged PM versus phagosomal cup

<table>
<thead>
<tr>
<th>Fluorescent moiety</th>
<th>PM Recovery</th>
<th>t_{1/2} for recovery</th>
<th>R^2</th>
<th>Phagosomal cup Recovery</th>
<th>t_{1/2} for recovery</th>
<th>R^2</th>
</tr>
</thead>
<tbody>
<tr>
<td>PM-GFP</td>
<td>110%</td>
<td>15</td>
<td>0.995</td>
<td>36%</td>
<td>8</td>
<td>0.990</td>
</tr>
<tr>
<td>PM-GFP + MβCD</td>
<td>110%</td>
<td>23</td>
<td>0.996</td>
<td>44%</td>
<td>10</td>
<td>0.988</td>
</tr>
<tr>
<td>PM-GFP + PP1</td>
<td>96%</td>
<td>12</td>
<td>0.996</td>
<td>76%</td>
<td>10</td>
<td>0.988</td>
</tr>
<tr>
<td>H-Ras-GFP</td>
<td>78%</td>
<td>10</td>
<td>0.998</td>
<td>35%</td>
<td>4</td>
<td>0.995</td>
</tr>
<tr>
<td>K-Ras-GFP</td>
<td>113%</td>
<td>9</td>
<td>0.997</td>
<td>90%</td>
<td>8</td>
<td>0.992</td>
</tr>
<tr>
<td>LAT-GFP</td>
<td>96%</td>
<td>6</td>
<td>0.995</td>
<td>99%</td>
<td>6</td>
<td>0.997</td>
</tr>
<tr>
<td>GPI-YFP</td>
<td>97%</td>
<td>8</td>
<td>0.994</td>
<td>99%</td>
<td>7</td>
<td>0.987</td>
</tr>
<tr>
<td>GPI-YFP + PP1</td>
<td>93%</td>
<td>6</td>
<td>0.995</td>
<td>85%</td>
<td>8</td>
<td>0.995</td>
</tr>
<tr>
<td>PS-NBD</td>
<td>75%</td>
<td>9</td>
<td>0.980</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>PE-BODIPE</td>
<td>93%</td>
<td>10</td>
<td>0.975</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
</tbody>
</table>
Figure 3.2: Photobleaching of PM-GFP at the phagosomal cup.

RAW264.7 cells were transfected with PM-GFP, and phagocytosis was initiated by addition of IgG-opsonised beads (8.3-μm diameter) while the cells were being analysed by differential interference contrast (DIC) and LSM. (A) DIC image. (B) Corresponding LSM transverse optical slice acquired before bleaching near the middle of the phagocytic cup. Bar, 5 um. (C) LSM transverse optical slice acquired shortly after bleaching the area indicated by the circle. (D) Course of FRAP. Bleaching was performed at the arrow. Two areas were bleached: one near the middle of the cup (open circles) and the other in an unengaged, contralateral area of the cell membrane (closed circles). Data are means ± SEM of eight individual experiments. Where absent, error bars were smaller than the symbol.
PM-GFP at the cup was markedly decreased (MF = 0.36 ± 0.01; n = 8). The diffusion rate of the remaining MF was indistinguishable from the PM-GFP in the bulk membrane. These findings suggest that the mobility of acylated proteins is diminished in the vicinity of the Fc-gamma receptors associated with and activated by the phagocytic particle. Phagosomes undergo membrane remodeling after sealing, a consequence of active fusion and fission events (Vieira et al., 2002). We found that for large beads such as those used in the present study, remodeling starts even before phagocytosis is completed (Figure 3.9). It was therefore important to ascertain that the failure of PM-GFP fluorescence to recover was a reflection of immobility and not its removal from the membrane. To this end, we performed careful quantitation of the rate of disappearance of PM-GFP and of another membrane marker, glycosylphosphatidylinositol (GPI)-anchored YFP during phagocytosis of large beads. Up to 40% of the fluorescence is lost from the base of the cup in 3 min (Figure 3.9). We therefore limited our FRAP experiments to the initial 100 s, when the loss by remodeling is modest.

**Mobility of other GFP constructs anchored to the inner monolayer:** Stimulatory Fc-gamma receptors bearing an immunoreceptor tyrosine-based activation motif associate with and become phosphorylated by Src-family kinases, including Lyn. Because the N-terminal sequence used to target PM-GFP to the membrane was derived from Lyn, we considered the possibility that a specific, direct interaction with the receptor complex might account for the reduced mobility of the fluorescent probe. As an alternative strategy to target GFP to the inner aspect of the PM, we attached the C-terminal 9 amino acids of H-Ras to GFP. In addition to the prenylation that is characteristic of all Ras isoforms, the C terminus of H-Ras is doubly acylated, and the chimeric GFP comprising the 9 amino acids of H-Ras (GFP-tH) undergoes the same posttranslational modifications (Apolloni et al., 2000). As a result, GFP-tH targets almost exclusively to the plasmalemma in BHK cells (Apolloni et al., 2000), as well as in RAW macrophages (Figure 3.3A). As shown in Figure 3.3B (solid squares) and Table 2, when photobleached in resting macrophages or in the unengaged region of cells engulfing beads, GFP-tH recovered rapidly (t1/2 = 10 ± 0.5 s; n = 8) and extensively (MF = 0.78 ± 0.01). In contrast, at the phagosomal cup, a sizable fraction of GFP-tH was immobile during the period analysed (MF = 0.37 ± 0.01; n = 8). The diffusion of the MF was similar to that of the un-engaged control membrane (Table 2). Therefore, two different acylated GFP probes displayed reduced mobility in nascent phagosomes. Because the C terminus of H-Ras is not anticipated to interact with Fc receptors, it
Figure 3.3: Photobleaching of GFP-tH.

RAW264.7 cells were transfected with GFP-tH, and phagocytosis was initiated by addition of IgG-opsonised beads while the cells were being analysed by DIC and LSM. (A) Confocal LSM image taken before bleaching, showing the distribution of GFP-tH. (B) Time course of FRAP of GFP-tH; bleaching was performed at the arrow. Two areas of the cell were bleached: one near the middle of the cup (open squares) and the other in an unengaged, contralateral area of the cell membrane (closed squares). Data in B are means ± SEM of eight individual experiments. Where absent, error bars were smaller than the symbol.
is unlikely that direct association with the receptor complex is responsible for the immobilisation of either probe.

**Role of the actin cytoskeleton in lipid mobility in nascent phagosomes:** The preceding data indicate that two different probes anchored to the cytosolic aspect of the membrane exhibit reduced mobility at sites of phagosome formation. One possible obstacle to the movement of inner membrane-associated GFP is the actin cytoskeleton. Accumulation of actin and other cytoskeletal proteins is a well-established feature of phagosome generation (Tse et al., 2003). We therefore considered whether the partial immobilisation of PM-GFP and GFP-tH at the cup resulted from steric hindrance by actin-associated proteins. It has been shown that preventing actin polymerisation with cytochalasin D does not abrogate bead engagement or the subsequent tyrosine phosphorylation of Fc receptors (Greenberg et al., 1994). In accordance with these findings, we found that when large opsonised beads were added to cytochalasin-treated cells, cup formation was evident (Figure 3.4, A and B), enabling us to perform FRAP with minimal actin polymerisation. In cells treated with cytochalasin D, the mobility of PM-GFP in the bulk membrane was unaffected (MF = 0.84; Table 2). However, the area of the membrane engaged in phagocytosis still showed reduced mobility (Figure 3.4C). To further study this phenomenon, we took advantage of the observation that under normal conditions actin dissociates from the membrane as the phagosome seals. The accumulation of F-actin at the base of the cup and its dissociation from the membrane of recently formed phagosomes is documented dynamically in Video 1 (available at http://www.jcb.org/cgi/content/full/jcb.200605044/DC1). Note that the amount of residual F-actin associated with formed phagosomes is minute, much lower than that of the unengaged PM. Because of the premature membrane remodeling observed during engulfment of large beads, smaller particles (3.1 μm) were used to ensure sufficient retention of the probe in sealed phagosomes (Figure 3.4E). We were thus able to compare the lateral mobility of PM-GFP in actin-depleted sealed phagosomal and PMs. As shown in Figure 3.4F, the MF of the probe in the sealed vacuole remained considerably lower than that of the bulk plasmalemma (MF = 0.26 ± 0.02 and 0.9 ± 0.02, respectively; n = 8). Qualitatively similar results were obtained using 8.3-μm beads, but the results were less reliable because of the small amount of fluorescence remaining after sealing (unpublished data). Given the small amount of actin that remains associated with the formed phagosome, it is unlikely that the reduced mobility of lipid-associated probes is attributable to physical hindrance by the cytoskeleton.
Figure 3.4: The effect of actin on the Mobile Fraction (MF) of PM-GFP in early and formed phagosomes.

(A and B) DIC and LSM images of PM-GFP respectively, showing the extent of development of phagocytic cups in RAW cells treated with cytochalasin D. Phagocytosis was initiated by the addition of IgG opsonised beads (8.3-μm diameter). (C) Recovery of PM-GFP fluorescence after photobleaching performed at the cup and an unengaged area of the membrane. (D–F) Photobleaching of PM-GFP in formed phagosomes. Phagocytosis was initiated by addition of IgG-opsonised latex beads (8.31-μm diameter), and after 6 min the cells were fixed and stained with labelled phalloidin to visualise F-actin by LSM (D). (E) Fluorescence of PM-GFP in the same cell stained for actin in D. Arrowheads in D and E indicate the location of three internalised beads. A typical area designated for bleaching is indicated by the circle. (F) Course of FRAP. Bleaching was performed at the arrow. Two areas of each cell were bleached: one in the phagosomal membrane (phago) and the other in the cell membrane (bulk). Data are means ± SEM of seven individual experiments. Error bars were smaller than the symbol.
Figure 3.4

[Description of Figure 3.4: Diagrams and graphs illustrating experimental results.]

A, B, C, D, E, F: Image panels showing different stages or conditions of the experiment.

C: Graph showing recovery percentage over time with and without Cytochalasin D.

E: Diagram with labeled areas indicating specific time points and cellular structures.

F: Graph with points plotted over time, indicating bulk and phago recovery percentages.
Mobility of GFP constructs anchored to the outer monolayer: Lipids containing saturated acyl chains are thought to localize preferentially in sphingolipid and cholesterol-enriched microdomains often called ‘rafts’ (Simons and Ikonen, 1997). Cross-linked Fc-gamma receptors are thought to cluster in similar microdomains (Kwiatkowska and Sobota, 2001). It therefore seemed likely that the reduced mobility of PM-GFP and GFP-tH could result from trapping in poorly mobile raft aggregates at the phagocytic cup. To test this hypothesis, we measured the lateral mobility of GPI-GFP and -YFP, as GPI-linked proteins partition selectively in rafts (Nichols, 2003). When expressed in macrophages, GPI-GFP is present largely in the plasmalemma (Figure 3.5A). That the protein is anchored to the outer monolayer was verified by its accessibility to anti-GFP antibodies added extracellularly to intact cells (unpublished data). Most of the GPI-anchored probe was mobile in resting cells (MF = 0.81 ± 0.04; n = 8) and in the unengaged regions of the membrane of cells performing phagocytosis (Table 2). The diffusion rate was similar to that of PM-GFP (Figure 3.5D). More important, the MF and rate of diffusion of GPI-GFP or -YFP were virtually identical at the phagocytic cup and elsewhere in the unengaged membrane (Table 2). Therefore, the behavior of GPI-anchored probes is distinctly different from that of PM-GFP and GFP-tH.

Role of microdomains in lipid mobility at the cup: It has recently become apparent that different types of lipid microdomains (rafts) can coexist in cells (Kusumi et al., 2004). Therefore, the differential behavior of GPI- and PM-GFP does not necessarily rule out raft involvement in reducing the mobility of PM-GFP. To further explore the role of lipid microdomains, we transfected cells with a lipid-anchored construct that is largely excluded from the rafts. GFP-tK was constructed by adding the C-terminal 17 residues of K-Ras to GFP. This portion of the hypervariable domain of K-Ras includes the prenylation CAAX box plus a polycationic sequence that directs the resulting chimera to anionic lipids of the cytosolic face of the plasmalemma (Apolloni et al., 2000). As anticipated, GFP-tK was predominantly found at the cell membrane (unpublished data). In unstimulated cells, as well as in unengaged regions of the membrane of cells performing phagocytosis, GFP-tK was highly mobile (Table 2; MF = 1.13 ± 0.03; t1/2 = 9 ± 1 s; n = 6). Its mobility was only marginally lower at the phagocytic cup (MF = 0.90 ± 0.03; t1/2 = 7.8 ± 1.4 s; n = 6). The differential behavior of the various lipid-associated proteins tested suggests that individual microdomains have distinct mobility within nascent
**Figure 3.5: Photobleaching of GPI-GFP at the phagosomal cup.**

RAW264.7 cells were transfected with GPI-GFP, and phagocytosis was initiated by addition of IgG-opsonised beads (8.3-µm diameter) while the cells were being analysed by differential interference contrast (DIC) and LSM. (A) LSM transverse optical slice acquired before bleaching near the middle of the phagocytic cup. Bar, 5 µm. (B) LSM transverse optical slice acquired shortly after bleaching the area indicated by the circle. (C) Corresponding DIC image. (D) Comparison of the course of FRAP in otherwise unstimulated cells transfected with either GPI- or PM-GFP. (E) Comparison of the course of FRAP of GPI-GFP at the cup (open circles) and in an unengaged region of the PM (closed circles). In D and E, bleaching was performed at the arrow and data are means ± SEM of eight experiments of each type. Where absent, error bars were smaller than the symbol.
phagosomes. To further test the role of lipid microdomains, we used methyl-$\beta$-cyclodextrin (M\(\beta\)CD) to remove cholesterol from the membrane (Klein et al., 1995). Cholesterol is essential for the formation of most lipid rafts, and its removal consistently leads to their destabilisation (Kwiatkowska et al., 2003). Treatment of RAW cells with M\(\beta\)CD as described in Materials and Methods resulted in sizable removal of plasmalemmal cholesterol, which could be readily visualised by staining the cells with filipin (Figure 3.6, A and B). The total cellular content of cholesterol, determined using a cholesterol oxidase-based spectroscopic assay, was reduced by 50% after treatment with M\(\beta\)CD. Of note, extraction of cholesterol did not affect the mobility of PM-GFP in otherwise untreated cells. The MF (1.27 ± 0.1; n = 7) and the diffusion rate (\(t_{1/2} = 23 ± 4\) s) were altered only marginally by pretreatment with M\(\beta\)CD (Figure 3.6D and Table 2).

Cholesterol depletion had no discernible effect on the ability of RAW cells to ingest opsonised beads, consistent with earlier findings (Peyron et al., 2000); note, however, that inhibitory effects of M\(\beta\)CD on some types of phagocytosis have also been reported (Peyron et al., 2000; Kwiatkowska and Sobota, 2001). More important, the immobilisation of a large fraction of PM-GFP at the phagocytic cup persisted in cholesterol-depleted cells (Figure 3.6, C and E; MF = 0.44 ± 0.02; n = 7). These findings imply that normal cholesterol content is not essential for the preservation of the microdomains that experience reduced mobility at sites of phagocytosis.

Further evidence that sphingolipid and cholesterol-rich microdomains do not mediate the immobilisation of diacylated proteins was obtained by studying the behavior of LAT (linker for activation of T cells). This adaptor is a transmembrane protein known to associate preferentially with such microdomains. As shown in Figure 3.6 F, GFP-tagged LAT localised to the PM, as reported for the native protein and for the fluorescent chimera in lymphoid cells (Bonello et al., 2004). Of note, the mobility of LAT at the phagocytic cup was not different from that measured elsewhere in the cell (Figure 3.6 G and Table 2). These observations suggest that cholesterol-enriched rafts are not noticeably immobilised in the vicinity of engaged Fc-gamma receptors.

**Tyrosine phosphorylation is required to reduce lipid mobility:** We next investigated whether the alteration in the mobility of lipid-associated proteins during phagocytosis is a passive consequence of receptor clustering at the cup or requires active signaling. Tyrosine phosphorylation of the Fc\(\gamma\) receptors by Src-family kinases is one of the earliest events in the
Figure 3.6: Effect of cholesterol depletion on the mobility of PM-GFP.

(A and B) Effect of MβCD on cholesterol content. RAW264.7 cells were incubated in the presence (B) or absence (A) of 10 mM M-beta-CD for 30 min, and their cholesterol content was observed by fluorescence microscopy after staining with filipin. (C) RAW264.7 cells transfected with PM-GFP were treated with or without MβCD as in A and B and then exposed to IgG-opsonised beads to initiate phagocytosis. The course of FRAP of PM-GFP is illustrated. Two areas were bleached: one near the middle of the cup (open squares) and the other in an unengaged area of the cell membrane (closed squares). (D) Comparison of the half-time for recovery of PM-GFP after bleaching in otherwise untreated (open bar) and in M-beta-CD-extracted cells (solid bar). (E) Comparison of the fractional recovery of PM-GFP after bleaching at the phagocytic cup (open bars) and in unengaged regions of the membrane (solid bars) in cells undergoing phagocytosis. The cells had been either untreated or extracted with MβCD as specified. Data in D and E are means ± SEM of seven determinations. (F) LSM transverse optical slice acquired before bleaching of RAW cells transfected LAT-GFP. (G) Course of FRAP after bleaching at the phagocytic cup (open symbols) and in unengaged regions of the membrane (closed symbols) in cells undergoing the phagocytosis of 8.3-µm beads. Bleaching occurred at the arrow, and data are means ± SEM of six experiments of each type. Where absent, error bars were smaller than the symbol.
signaling cascade and is essential for progression of phagocytosis. Inhibition of Src-family kinases with inhibitors such as PP1 and PP2 precludes particle internalisation (Majeed et al., 2001; Song et al., 2004), yet does not prevent receptor-ligand association and formation of a well-defined phagocytic cup (Figure 3.10, B and E). We were therefore able to assess the mobility of both PM-GFP and GPI-YFP at the cup of PP1-inhibited cells. The effectiveness of the kinase inhibitor was verified by its ability to prevent the PLC-mediated hydrolysis of PtdIns(4,5)P2 (measured using a specific pleckstrin homology [PH] domain; Figure 3.10, B and E), a tyrosine phosphorylation-dependent event, and by the virtually complete inhibition of particle engulfment despite the formation of stable incipient cups. As illustrated in Figure 3.7, the mobility of the lipid-associated probe was only marginally reduced at the cup, compared with the bulk, unengaged membrane. In eight experiments using PM-GFP, the MF was 0.76 ± 0.03 in the former and 0.96 ± 0.03 in the latter. Similarly, the fraction and half-time of GPI-YFP recovery in the presence of PP1 were not significantly altered (Figure 3.7 and Table 2). The possible role of phosphatidylinositol 3-kinase in controlling lipid mobility was also investigated. We initially confirmed that under the conditions used, the inhibitor LY294002 impaired phosphatidylinositol 3-kinase activity, as it prevented the accumulation of 3'-phosphorylated polyphosphoinositides normally observed at the cup during the early stages of phagocytosis (Figure 3.10, C and F). As reported earlier (Araki et al., 1996; Cox et al., 1999), treatment with inhibitors of this kinase arrested the development of phagosomes at an intermediate stage, where cup formation is evident but sealing is impaired, particularly in the case of large beads such as those used in this study (Figure 3.10, C and F). As expected, LY294002 had no discernible effect on the mobility of the exofacial marker GPI-YFP. Interestingly, the immobilisation of PM-GFP normally seen in untreated cells persisted in the presence of the inhibitor (MF = 0.46), as illustrated in Figure 3.7 and quantified in Table 2. These findings imply that generation of 3'-phosphorylated inositides is not essential to retain acylated molecules in the inner aspect of the phagocytic cup.

3.4 Discussion

Suitability of the probes used: The objective of our experiments was to assess the mobility of lipid-associated proteins in the phagosome and to define its determinants. The fluorescent proteins used in this study, such as PM-GFP and GFP-tH, are suitable probes to measure the
Figure 3.7: Effect of kinase inhibitors on the mobility of inner and outer membrane leaflet probes

RAW cells transfected with PM-GFP (A and C) or GPI-GFP (B and D) were pretreated with either 10 μM PP1 for 1 h (A and B) or 100 μM LY294002 for 30 min (C and D) and then exposed to 8.3-μm IgG-opsonised beads to initiate phagocytosis. PP1 or LY294002 were maintained in the medium during phagocytosis. The course of FRAP is illustrated. Two areas were bleached: one near the middle of the cup (open squares) and the other in an unengaged area of the cell membrane (closed squares). Data are means ± SEM of eight determinations. Where absent, error bars were smaller than the symbol.
Figure 3.7
contribution of the hydrophobic moiety of lipid-anchored molecules such as diacylated Src-family kinases and small GTPases, which are critical for the onset and development of phagocytosis. The molecular weight, acyl chain composition, and membrane disposition of the probes is very similar to that of the endogenous signaling molecules, yet they greatly simplify the analysis by obviating protein-protein interactions. In addition, acylated fluorescent proteins are arguably good models to analyse the mobility of lipids in the plane of the bilayer. At first glance, it may appear that attachment of the comparatively large protein moiety to the acyl chains would greatly reduce the lateral mobility of the complex, compared with that of endogenous phospholipids. However, hydrophobic interactions within the bilayer appear to be, by far, the main impediment to the lateral displacement of lipids and lipid-anchored proteins. We calculated diffusion coefficients of $\sim 2.3 \times 10^{-10}$ cm$^2$/s for labelled phospholipids and $1.5 \times 10^{-10}$ cm$^2$/s for diacylated GFP in RAW cells. Such coefficients are nearly three orders of magnitude lower than that reported for GFP in water ($9 \times 10^{-7}$ cm$^2$/s), which is reduced only three- to four fold when the protein is expressed in intracellular compartments, including the cytosol (Partikian et al., 1998; Chen et al., 2002). Therefore, attachment of a GFP moiety would be expected to contribute minimally to the mobility of the acyl chains in the plane of the membrane.

**Reduced mobility of lipid-associated proteins at the phagocytic cup:** The main observation reported in this paper is that the mobility of lipid-attached probes differs in the phagocytic cup from that in the bulk of the unengaged membrane. We considered whether fission of endocytic vesicles, too small to be detected by the optical microscope, accounted for the failure of the fluorescence to recover at the cup. Indeed, remodeling of the cup formed during ingestion of large beads commenced before sealing (Figure 3.9). To minimize the confounding effect of this remodeling, all our experiments were performed at the onset of phagocytosis and limited to the first 100 s after bead engagement. More important, although all the lipids analysed underwent parallel remodeling (Figure 3.9), only PM-GFP and GFP-tH displayed reduced mobility. GPI-anchored probes, which are remodeled at a similar rate, recovered to a much greater extent, as did GFP-tK. Jointly, these considerations rule out endocytosis as a viable explanation for the incomplete fluorescence recovery. The differential behavior of GPI-YFP, a probe located on the outer monolayer of the plasmalemma, and PM-GFP, an inner monolayer probe, could be attributed to a unique steric hindrance on the cytosolic face of the membrane. The actin cytoskeleton would be an obvious candidate for such a physical obstacle. Cytoskeletal proteins
may restrict the motion of the GFP moiety and in extreme cases, corral it within domains that are fenced in. Several lines of evidence argue against this. First, although actin and its associated proteins do indeed accumulate at the base of the cup during the initial stages of phagocytosis, they subsequently detach. In fact, in the case of large beads such as those used in our experiments, the density of actin below the forming phagosome drops below the levels of the unengaged membrane, where the mobility of the lipid-associated probe remains high. Moreover, lipid immobilisation persisted in the region subtending the particle in cells treated with cytochalasin D to preclude actin polymerisation. Moreover, in the absence of the inhibitor, an extreme situation is reached after the phagosome seals, when actin is no longer detectable on its membrane, yet the reduction in lipid mobility persisted (Figure 3.4). Second, the mobility of GFP-tK was altered much less than that of GFP-tH or PM-GFP (Table 2). Because the size and disposition of the protein moiety of the probes with respect to the membrane are similar in all cases, fencing in by cytoskeletal elements is an unlikely explanation for the altered mobility. Still, a contribution of the actin network to the mobility of the probes cannot be entirely ruled out. If it exists, such a steric hindrance would contribute little to the mobility of free lipids but would nevertheless affect lipid-associated proteins, whether the association is covalent, as in the case of Src-family kinases, or electrostatic, as in the case of proteins bearing PH domains.

We believe that immobilisation of lipid microdomains in the vicinity of clustered Fc-gamma receptors is the most likely explanation for our observations. Lipid rafts, cholesterol-rich microdomains that also contain glycosphingolipids, are often invoked in the context of signal transduction by immunoreceptors (Magee et al., 2002). Both PM-GFP and GFP-tH would be expected to partition into such rafts, and coalescence of the latter around activated receptors may have contributed to immobilisation of the probes. However, our observations do not fit the conventional model of the raft on two accounts. First, the mobility of GPI-YFP, which is predicted to reside in rafts, was similar in the cup and elsewhere in the membrane. Second, extraction of 50% of the total cellular cholesterol and likely an even greater fraction of the plasmalemmal cholesterol had little effect on the immobilisation of PM-GFP at the cup. Two explanations can be considered: (1) that coalescence of lipid microdomains is not the mechanism underlying the change in lipid mobility and (2) that unique microdomains that do not conform to the conventional sphingolipid and cholesterol-rich raft are responsible. In this regard, it is noteworthy that unlike the situation reported for immunoreceptors in lymphoid and basophilic
cells (Vereb et al., 2000; Surviladze et al., 2001), extraction of cholesterol does not impair Fc-
gamma receptor signaling, leading to phagocytosis (Gatfield and Pieters, 2000; Peyron et al.,
2000).

Passive coalescence of lipid microdomains cannot explain the sensitivity of the immobilisation to
inhibitors of Src- family kinases, which provide the earliest signal in the phagocytic cascade. We
propose that events that follow tyrosine phosphorylation contribute to the assembly of lipid
microdomains. It is conceivable that the recruitment of adaptor molecules with lipid-interacting
moieties facilitates the coalescence of specific lipids, thereby reducing their mobility. Two types
of adaptors could fulfill this function by different mechanisms. Transmembrane molecules that
preferentially associate with saturated lipids such as LAT can be recruited to activated receptor
complexes (Ragab et al., 2003). The lipid annulus associated with LAT or similar adaptors could
interact with and reduce the mobility of lipids with saturated chains in the immediate vicinity of
the activated receptor complex. Of note, the mobility of LAT itself has been documented to be
reduced when T cell receptors are stimulated (Tanimura et al., 2003). However, LAT is not an
essential adaptor of Fc receptors in phagocytes, which use other adaptors such as Gab2, GRB2,
and CrkII. Accordingly, we found that LAT is not recruited or immobilised at sites of
phagocytosis. Other, unidentified transmembrane adaptors may nevertheless cause the
immobilisation of selected lipids near the activated receptors, but soluble adaptors could
simply be involved. Adaptors bearing lipid binding domains would be recruited to the receptor
complex by the former and would stabilize lipids in its vicinity. Several adaptors possessing PH,
ENTH (epsin N-terminal homology), or VHS (Vps27, Hrs, and Stam) domains are known to
exist, and some of these, such as Gab2, have been reported to associate with Fc-gamma receptors
(Gu et al., 2003). Some signaling molecules, such as Vav or PLCδ, become part of the activated
receptor complex and contain lipid binding PH domains. Together, the proteins that cluster
around activated receptors can cause immobilisation of defined lipids. Importantly, the acyl
moieties of phosphoinositides would facilitate accumulation and immobilisation of other lipids
with saturated chains and of proteins like diacylated Src-family kinases or GTPases.

In summary, we propose a model whereby clustering and activation of Fc-gamma receptors may
lead to the recruitment and stabilisation of specific lipids and/or lipid-associated proteins in the
active zone. Adaptors or other signaling molecules that become recruited to the signaling
complex may induce the formation of a defined lipid annulus, effectively a microdomain that
need not be stabilised by cholesterol but by hydrophobic interactions between the saturated acyl chains of its constituents. Such stabilisation would have critical consequences for localised signaling, restricting the diffusion of phosphoinositides and focally attracting adaptors and transducers.

3.5 Materials and Methods

**Reagents**: Polystyrene beads (3.1 and 8.3 μm in diameter) were obtained from Bangs Laboratories. FuGene 6 was purchased from Roche Molecular Biochemicals. PP1 was obtained from BIOMOL Research Laboratories, Inc., LY294002 from Calbiochem, and alpha-MEM from Wisent, Inc. Cy3-labelled secondary antibodies were obtained from Jackson ImmunoResearch Laboratories. Rhodamine-phalloidin and the Amplex red cholesterol assay kit were obtained from Invitrogen. Human IgG, M-beta-CD, filipin, and all other reagents were obtained from Sigma-Aldrich. The headgroup-labelled lipids nitrobenzoxadiazole (NBD)-phosphatidylserine and boron dipyrromethene difluoride (BODIPY)-phosphatidylethanolamine were purchased from Avanti Polar Lipids, Inc., and Invitrogen, respectively. The synthetic medium used for fluorescence determinations consisted of 140 mM NaCl, 3 mM KCl, 10 mM glucose, 20 mM Hepes, 1 mM MgCl₂, and 1 mM CaCl₂, pH 7.4 (290 ± 5 mosM).

**cDNA constructs**: PM-GFP encodes the 10 amino acid myristoylation/palmitoylation sequence from Lyn fused to enhanced GFP (Teruel *et al.*, 1999). GFP-tH consists of the C-terminal 9 amino acids of H-Ras fused to the C terminus of GFP. GFP-tK consists of the C-terminal 17 amino acids of K-Ras fused to the C terminus of GFP. These C-terminal regions comprise the complete targeting domains of H- and K-Ras, respectively (Apolloni *et al.*, 2000). GPI-GFP and -YFP encode the 26 amino acid signal sequence of insulin fused to enhanced GFP or YFP, followed by the 43 amino acid GPI sequence motif of decay-accelerating factor (Kenworthy *et al.*, 2004). Construction of the LAT-GFP plasmid was detailed in Bonello *et al.* (2004). For localisation of PtdIns(4,5)P₂ and PtdIns(3,4,5)P₃, we used enhanced GFP fusions of the PH domains of PLCδ or Akt, respectively, both gifts from T. Meyer (Stanford University, Stanford, CA).

**Cell culture, transfection, and phagocytosis**: The macrophage RAW264.7 cell line was obtained from American Type Culture Collection. These macrophages, referred to hereafter and throughout as RAW cells, were cultured in alpha-MEM supplemented with 10% fetal calf serum
at 37°C under a humidified 5% CO₂ atmosphere. Cells were trypsinised and seeded onto 2.5-cm glass coverslips at ~30% confluence. Cells were transiently transfected by lipofection using FuGene 6 or by electroporation with the Nucleofector system (Amaxa) according to the manufacturers’ directions and used within 16-24 h of transfection. Polystyrene beads were opsonised with 1 mg/ml human IgG by incubation for at least 1 h at 37°C, followed by three washes with PBS. To initiate phagocytosis, opsonised beads were allowed to sediment on RAW cells grown on coverslips and bathed in synthetic medium at 37°C.

**Loading of fluorescent lipids:** RAW macrophages were grown on glass coverslips to ~30% confluence. 50 μl of either lipid (dissolved in chloroform) were added to 9.5 ml of synthetic medium supplemented with 1 ml bovine serum albumin and mixed vigorously. Cells were overlaid with the lipid suspension and incubated at 4°C for 60 min. Cells were then washed and warmed with medium at 37°C before measuring fluorescence.

**Fluorescence determinations:** The distribution and mobility of the fluorescent chimeras was analysed by confocal microscopy. Laser-scanning confocal microscopy was performed with a LSM510 system (Carl Zeiss MicroImaging, Inc.) using a 100x (1.4 NA) oil-immersion objective lens. The standard laser excitation line (488 nm) and emission filter (543 nm) were used to image GFP and YFP-tagged chimeras. Coverslips bearing transfected cells were transferred to a thermostatted Leiden chamber holder on the microscope stage, where they were maintained at 37°C. For measurements of FRAP, two areas of 2 μm in diameter, one at the phagosomal cup and a second one at an unengaged portion of the plasmalemma of the same cell were defined. After acquiring three basal readings, the selected regions were irreversibly photobleached by repeated exposure to the 488-nm laser line set at 100% power. Under the conditions of our experiments (18 mW power output), nearly complete bleaching required 60 iterations, a process that was completed in 1-2 s. The recovery of fluorescence was then monitored over time by scanning the bleached area at the conventional (low) laser power to minimize photobleaching during sampling. To determine the size and shape of the bleached area, cells were fixed with 4% paraformaldehyde for 1 h before mounting in the Leiden chamber, to prevent lateral mobility of the tagged proteins and recovery of fluorescence. Photobleaching was then performed as described in the previous paragraph, and fluorescence images were obtained throughout the height of the cell by optical sectioning using the confocal microscope. Three-dimensional
reconstruction of the stacked images using the LSM510 software enabled us to reconstruct the pattern of the bleached area.

**FRAP data analysis:** To analyse the rate of recovery, we compared the fluorescence of the bleached area to that of an adjacent unbleached area of the same cell with similar fluorescence intensity. For each time point, the fluorescence of the bleached area was normalised to that of the corresponding control (unbleached) area to correct for possible drift of the focal plane or photobleaching incurred during the low-light sampling. For reference, images of the entire field were acquired immediately before and at the end of the experiment. All FRAP measurements were performed at 37°C. Data were fit to a simple diffusion, zero flow model (Yguerabide et al., 1982) using the formula

\[ F(t) = \frac{F_{(t=0)} + F_{(t=\infty)} \times (t/t_{1/2})}{1 + (t/t_{1/2})} \]

where the fluorescence intensity \( F \) at a given time \( t \) is related to the maximal fluorescence \( F_{(t=\infty)} \) and the half-time of maximal recovery \( t_{1/2} \). Using this equation, recovery curves were fit by least squares using Prism 4 (GraphPad Software, Inc.). In all cases, this method provided highly significant \( R^2 \) values that were comparable to and generally larger than those obtained using other models for single or multiple components of diffusion or flow (Yguerabide et al., 1982; Lippincott-Schwartz et al., 1999). Diffusion coefficients were calculated from the \( t_{1/2} \) of the recovery curves as previously described (eq. 19 in Axelrod et al., 1976).

**Actin staining and manipulation:** To label F-actin, cells were washed twice with PBS and fixed with 4% paraformaldehyde for 1 h. The cells were next permeabilised in 0.1% Triton X-100 and 100 mM glycine in PBS for 10 min and stained with a 1:400 dilution of rhodamine-phalloidin for 1 h. Where specified, actin polymerisation was impaired by pretreating the cells with 2 \( \mu M \) cytochalasin D for 10 min at 37°C as previously described (Greenberg et al. 1994). The inhibitor was maintained in the medium throughout the fluorescence determinations.

**Cholesterol manipulation and determination:** Where indicated, the cells were incubated with 10 mM MβCD for 30 min at 37°C to remove cholesterol. To verify the effectiveness of the treatment, cells were then fixed with 4% paraformaldehyde and incubated with 25 \( \mu g/ml \) of filipin, a cholesterol binding fluorescent probe, in PBS containing 1 mM MgCl2 and CaCl2 for
1h at 4°C. Cells were then washed twice with PBS, and images were acquired with excitation at 488 nm and emission at 500-550 nm. More quantitative estimates of cholesterol content were made using the Amplex red cholesterol assay kit, according to the manufacturer’s instructions.

**Pharmacological inhibition of kinases:** To inhibit phosphatidylinositol 3-kinases or Src-family kinases, RAW cells were exposed to either 100 μM LY294002 for 30 min or 10 μM PP1 for 1 h, respectively, at 37°C before the fluorescence determinations. Inhibitors were maintained in the medium throughout the microscopy experiments.

### 3.6 References


3.7 Supplementary Figures

Figure 3.8: Estimation of the time required for the complete phagocytosis of polystyrene beads of different sizes by RAW cells.

(A) Phagocytosis was initiated by addition of IgG-opsonised beads of either 3.1 or 8.3 μm (as specified), and DIC images were acquired at the indicated times. Time for internalization was measured from the point of engagement to pseudopod fusion and is quantified in B. Data are means ± SEM of three separate experiments, each involving >10 events.
Figure 3.8

A

Bead Size (μm)

Bead Size (μm)

Internalization time (min)

90 s

360 s

60 s

240 s

0 s

3.3

8.3
Figure 3.9: Persistence of inner and outer leaflet membrane probes during phagocytosis.

RAW cells transfected with either PM-GFP or GPI-YFP were exposed to IgG-opsonised beads to initiate phagocytosis. The relative fluorescence intensity of the base of the cup relative to that of the bulk membrane was monitored throughout entire phagocytic events. Representative images obtained in a PM-GFP transfected cell immediately after contact of the bead and 4 min after initiation of phagocytosis are shown in A and B, respectively, and a detailed time course is summarised in C. Asterisks denote the position of the bead. Data in C are means ± SEM of three experiments for each probe. The typical period of time used for FRAP experiments (~90-100s after contact with the bead was established) is shown.
**Figure 3.10: Distribution of phospholipid probes in the presence of kinase inhibitors.**

RAW cells transfected with a GFP fusion of the PH domain of PLCδ (A-C) or Akt (D-F), to reveal the distribution of PtdIns(4,5)P₂ and PtdIns(3,4,5)P₃, respectively. Cells were left untreated (A and D) or were treated with the Src-family kinase inhibitor PP1 (B and E) or with the PI 3-kinase inhibitor LY294002 (C and F). In all cases, phagocytosis was initiated by the addition of 8.3-μm IgG-opsonised beads. Asterisks denote the positions of engaged beads.
Figure 3.10

LY294002

C

F

PP1

B

E

Control

A

D

PLCγ-PH GFP

AKT-PH GFP

*
Chapter 4

4 Actin dynamics at the phagocytic cup: control of depolymerisation by phosphatidylinositol 3-kinase

4.1 Abstract

The dynamics of actin remodeling during phagocytosis was studied by a combination of total interference reflection and spinning disc confocal microscopy. In addition to the well-documented phase of active polymerisation, a secondary wave of F-actin disassembly was detected. Disassembly was first apparent at the base of the phagocytic cup and subsequently spread, trailing the F-actin-rich advancing pseudopods. Prevention of actin dissociation with jasplakinolide impaired completion of phagocytosis. Actin clearance from the phagosomes was accompanied by inactivation of Rac/Cdc42, which was in turn associated with detachment from the membrane of Rac/Cdc42 guanine nucleotide-exchange factors (GEFs). GEF detachment was caused by the localised disappearance of phosphatidylinositol 4,5-bisphosphate and by dephosphorylation of phosphotyrosines. Strikingly, phosphatidylinositol 3-kinase activity was required for both dephosphorylation and for phosphatidylinositol 4,5-bisphosphate hydrolysis and, consequently for actin disassembly. We propose that actin disassembly from the sites of early receptor engagement is required to recycle actin or determinants of its assembly to the advancing pseudopods. Failure to recycle such components may limit the progression of the phagocytic cup, accounting for the observation that phosphatidylinositol 3-kinase inhibitors block phagocytosis of large, but not of small particles.

4.2 Introduction

Engulfment of pathogens by phagocytic cells is an essential component of the innate immune response. Phagocytosis is also central to tissue homeostasis and remodelling, as it mediates the clearance of apoptotic bodies. Unlike conventional endocytosis, phagocytosis is driven by and therefore strictly dependent on actin remodeling. Though various mechanisms have been invoked, which vary according to type of receptor engaged (Newman et al. 1991), it is generally agreed that phagocytosis requires de novo polymerisation of actin initiated by Rho-family GTPases. In the case of Fcγ receptor-initiated phagocytosis, actin drives the extension of pseudopods that encircle and ultimately engulf the target particle (Swanson & Hoppe, 2004).
Active actin polymerisation persists throughout the particle internalisation process. Indeed, vestiges of the recently polymerised actin are often visible at the site of phagosomes sealing, where a contractile acto-myosin “purse-string” is thought to contribute to scission of the vacuole from the plasma membrane (Swanson et al. 1999). Immediately after sealing, however, phagosomes are largely devoid of actin, rapidly attaining levels below that of the basal (unstimulated) plasma membrane. Loss of actin from the vacuolar membrane facilitates its fusion with endomembranes, thereby enabling phagosome maturation. However, as documented in more detail here, clearance of actin from nascent phagosomes begins prior to sealing, even as actin continues to polymerise at the tips of advancing pseudopods. Depolymerisation of actin becomes readily apparent at the base of the phagocytic cup, especially when large particles are employed. While its implications are not fully understood, this early phase of clearance may: a) allow recycling of actin and its binding partners to advancing sites of active polymerisation; b) enable the membrane to attain the curvature required to envelop the particle and/or c) permit focal fusion of endomembranes that contribute to pseudopod extension and to the early steps of maturation (Lee et al. 2007b).

While much has been learned about the events that trigger actin polymerisation during phagocytosis, much less is known about its termination. In this manuscript, we established improved experimental models to characterize the early phase of actin clearance and analysed the underlying mechanism.

4.3 Results

Actin clears from the base of the phagocytic cup: Murine macrophage (RAW264.7) cell lines stably expressing fluorescently labelled actin were used to dynamically monitor cytoskeletal remodeling. Similar results were obtained using lines transfected with GFP-actin or with mCherry-actin, suggesting that neither the fluorescent tag nor clonal differences influenced the outcome of the experiments. Accordingly, the different stages of actin redistribution identified by imaging the fluorescent live cells were replicated by transiently transfecting the cells with the calponin-homology domain of utrophin, a probe for F-actin (Burkel et al. 2007) or by fixing untransfected cells at suitable intervals and staining actin with phalloidin (Figure 4.7).
To better resolve the different regions of the forming phagosomal cup, two different systems were used. First, we used spinning-disc confocal microscopy to analyse macrophages engulfing large (8.3 μm diameter) particles. Polystyrene beads opsonised with IgG were used as the phagocytic target, preferentially engaging Fcγ receptors (FcγR) on the surface of RAW264.7 cells (referred to hereafter as RAW cells). As shown in Figure 4.1A, actin accumulates at the base of the phagosomal cup shortly after particle attachment. Subsequently, pseudopods extend, surrounding the bead. Actin is prominent at the leading edge of the pseudopods, but its density at the initial contact site, the base of the phagosomal cup, decreases markedly (Figure 4.1B). At the time of scission (Figure 4.1C) actin is only detectable at the junction between the phagosome and plasmalemma, while the rest of the vacuole is largely devoid of actin.

It is conceivable that refraction of light through the large latex bead affected our ability to detect actin at the base of phagosomes. A second experimental system was developed to rule out this possibility. The model consists of suspended RAW cells that are allowed to sediment onto IgG-coated coverslips (Figure 4.1D; see Methods for details). As FcγR are engaged, the cells emit pseudopods along the IgG-coated surface in a vain attempt to engulf it. Though eventually frustrated, the initial response is a valid facsimile of the phagocytic process. Because pseudopod spreading occurs on the plane of the coverslip, the fluorescence of submembranous actin can be imaged with excellent signal-to-noise ratio using total internal reflection fluorescence (TIRF) microscopy. Moreover, the entire sequence occurs at a single optical plane, with constant refractive index. As shown in Figure 4.1E-G, the improved optical features of the system enabled us to identify new features of the actin rearrangement process: at early stages (Figure 4.1E) actin accumulates densely throughout most of the contact area, as described for the beads. As the attempted phagocytosis proceeds (Figure 4.1F) a rim of actin is detected at the edge of the advancing pseudopods, while a discontinuous, punctate band of actin is found adjacent. At this stage the center of the frustrated phagocytic cup has lost a large fraction of the adherent actin, with only occasional puncta detectable. As the pseudopods spread further (Figure 4.1G) the punctate band becomes more discontinuous and actin is largely lost from the middle of the frustrated phagocytic “cup”. The differences in the actin density along the adherent surface can be quantified by line scanning (Figure 4.1H) and are also apparent in vertical reconstructions of sequential optical slices obtained by confocal microscopy (Figure 4.1I). The similarity of the
Figure 4.1: Clearance of actin at the phagocytic cup

RAW cells stably transfected with mCherry-actin were challenged with IgG-opsonised 8.3 μm beads. Images are typical of actin distribution 3 (A), 5 (B) or 8 min (C) after particle engagement. Asterisks indicate the position of the bead, monitored in parallel by DIC imaging (not shown). (D) Schematic of the frustrated phagocytosis model. RAW cells were suspended and then allowed to settle onto IgG-coated coverslips, whereupon they spread onto the coverslips while attempting to engulf the opsonised surface. (E-G) Representative images of a cell transfected with GFP-actin and imaged with TIRF microscopy 1 min (E), 3 min (F) and 4 min (G) after making contact with the opsonised surface. (H) Fluorescence intensity scan of the dashed line in (G); data were normalised to the highest intensity. (I) Serial optical slices of a cell at a stage like that illustrated in (G) were acquired using a spinning disc confocal microscope. A z vs. x (sagittal view) reconstruction is illustrated. (J) Schematic representation of the anticipated effect of jasplakinolide. Unlike untreated cells, which undergo actin clearance from the base of the cup (inset), cells treated with jasplakinolide are expected to retain actin throughout the phagocytic cup (main panel). (K) Representative image of an mCherry-actin transfected cell treated with 1 μM jasplakinolide 3 min after engagement of a bead. Image was acquired 7 min after engagement of the bead. (L) Effect of jasplakinolide on phagocytic efficiency. The phagocytic index, expressed as beads internalised per cell, was determined in cells treated with or without jasplakinolide. Data are means ± SE of at least 3 experiments, each counting at least 20 cells. All scale bars = 5 μm.
Figure 4.1
results obtained with the bead and frustrated phagocytosis models validates the concomitant occurrence of peripheral actin polymerisation and central disassembly during the advanced stages of phagosome formation.

**Actin clearance is required for completion of phagocytosis:** We next assessed whether actin clearance from the base of the phagocytic cup is essential for successful phagocytosis. To this end we used jasplakinolide. When used for short periods, this cell-permeant drug stabilizes pre-existing actin filaments, by binding to adjacent monomers in a filament. As shown diagrammatically in Figure 4.1J, we anticipated jasplakinolide to prevent or at least delay the depolymerisation of actin from the site where the cup originated. This prediction was indeed fulfilled: cells treated with the drug 3 min after initiation of phagocytosis displayed robust and sustained actin accumulation at the site of contact with the particle (Figure 4.1K). Strikingly, despite their enhanced ability to accumulate actin, the incipient cups failed to progress, resulting in a marked reduction in phagocytic efficiency (Figure 4.1L). Therefore, actin disassembly appears to be a critical component of phagosome formation.

**Termination of Rho-family GTPase activity accounts for actin clearance:** While being assembled, actin filaments continuously undergo disassembly and fragmentation, a process known as “treadmilling”. Because of the ongoing disassembly, cessation of polymerisation could account for the clearance of actin at the base of the cup. Because Cdc42 and Rac are required for FcγR-mediated polymerisation of actin (Cox et al. 1997), we studied the course of activation of these GTPases during phagocytosis using PAK1-PBD-GFP. As described earlier by Swanson and colleagues (Hoppe & Swanson, 2004), this fluorescent probe was recruited to the initial site of particle engagement (Figure 4.2A), coinciding with early sites of actin accumulation. PAK-PBD-GFP progressed along the sides of the bead as the pseudopods extended (Figure 4.2A’). Notably, the probe was depleted from the base of the cup, implying deactivation of Cdc42 and/or Rac in this region. Thus, abrogation of the activity of these GTPases could account for the loss of actin from the sites of early receptor engagement.

**Role of nucleotide exchange factors in the termination of Rac/Cdc42 activity:** The identity of the Guanine-nucleotide Exchange Factors (GEFs) that control the activity of Rac and Cdc42
**Figure 4.2: Localisation of active small GTPases and GEFs during phagocytosis**

RAW cells were transiently transfected with constructs encoding GFP-labelled (A) PAK-PBD, (B) Tiam1, (C) Vav1 or (D) DOCK180 and challenged with 8.3 \( \mu \text{m} \) IgG-opsonised particles. Representative images acquired 3 min (A–D; early phagocytosis stage) or 6 min (A’–D’; mid-phagocytosis stage) are illustrated. Asterisks denote the position of the bead, monitored in parallel by DIC imaging (not shown). Scale bars = 5 \( \mu \text{m} \)
<table>
<thead>
<tr>
<th>Protein</th>
<th>Early</th>
<th>Mid</th>
</tr>
</thead>
<tbody>
<tr>
<td>PAK-PBD</td>
<td><img src="A" alt="Image" /></td>
<td><img src="A'" alt="Image" /></td>
</tr>
<tr>
<td>Tiam1</td>
<td><img src="B" alt="Image" /></td>
<td><img src="B'" alt="Image" /></td>
</tr>
<tr>
<td>Vav1</td>
<td><img src="C" alt="Image" /></td>
<td><img src="C'" alt="Image" /></td>
</tr>
<tr>
<td>DOCK180</td>
<td><img src="D" alt="Image" /></td>
<td><img src="D'" alt="Image" /></td>
</tr>
</tbody>
</table>

Figure 4.2
during phagocytosis remains the subject of debate. Vav and DOCK180 have been implicated (Lee et al. 2007a, Patel et al. 2002), but not shown to be absolutely required. Because of the existing uncertainty, we analysed not only these GEFs but also the less well studied yet ubiquitously expressed Tiam1 and P-Rex1. We found no evidence that P-Rex1 was recruited to sites of phagocytosis (see Figure 4.8), and this GEF was therefore not explored further. By contrast, Tiam1, DOCK180 and Vav1 were all found to be present at sites where nascent phagosome formed. As shown in Figure 4.2B, Tiam1 was constitutively associated with the plasmalemma and therefore present at the time of receptor engagement. In contrast, Vav1 and DOCK180 were mostly cytoplasmic in unstimulated cells, but were recruited to sites of phagocytosis. Remarkably, all three GEFs lined the early phagocytic cup (Figure 4.2B-D) and at later stages they were all depleted from the base of the cup, while accumulating near the tip of the pseudopods (Figure 4.2B’-D’). Detachment of the GEFs from the cup can therefore readily account for the abrogation of Rac/Cdc42 activity.

Phospholipid metabolism directs Tiam1 dissociation from the membrane: The N-terminal pleckstrin homology (PH) domain of Tiam1 was found to be required for its constitutive association with the membrane (Ceccarelli et al. 2007). Because PH domains mostly interact with phosphoinositides and these lipids are actively metabolised at sites of phagocytosis (Botelho et al. 2000), we analysed whether phospholipid metabolism influenced Tiam1 distribution. We initially determined whether phosphatidylinositol (4,5)-bisphosphate (PtdIns(4,5)P2) is the PH domain ligand that retains Tiam1 at the membrane. To this end, cells were co-transfected with GFP-tagged Tiam1 and PLCδ-PH-mRFP, a probe for PtdIns(4,5)P2. As shown in Figure 4.3A and B, the two fluorophores were comparably concentrated in the inner aspect of the plasma membrane, consistent with the concept that PtdIns(4,5)P2 directs Tiam1 to the membrane. This notion was reinforced when the cells were treated with ionomycin. As shown earlier (Yeung et al. 2006) the elevation in cytosolic calcium induced by the ionophore activated phospholipase C, producing extensive degradation of PtdIns(4,5)P2, indicated by the dissociation of PLCδ-PH-mRFP from the membrane (Figure 4.3C). In parallel, Tiam1 also detached from the membrane (Figure 4.3D).

Figure 4.3E-F shows that hydrolysis of PtdIns(4,5)P2 can similarly account for the dissociation of Tiam1 from the cup during phagocytosis. As described earlier (Botelho et al. 2000)
Figure 4.3: Phospholipids regulate the localisation of Tiam1

RAW cells were doubly transfected with GFP labelled Tiam1 (B,D,F) and the PH domain of PLCδ labelled with mRFP (A,C,E). Cells were left unstimulated (A and B), were treated with 10 μM ionomycin (C and D) or challenged with 8.3 μm IgG-opsonised particles (E and F). Scale bars = 5 μm. Asterisks denote the position of the bead, monitored in parallel by DIC imaging (not shown).
Figure 4.3
PtdIns(4,5)P₂ is lost from the membrane during phagocytosis and this change is remarkably confined to the phagocytic cup (Figure 4.3E). The parallel behavior of PLCδ-PH-mCherry and Tiam1 (Figure 4.3F) strongly suggests that hydrolysis of PtdIns(4,5)P₂ dictates the dissociation of the GEF from the cup.

**Dephosphorylation contributes to Rac/Cdc42 deactivation:** Unlike Tiam1 that is constitutively present at the membrane, Vav1 and DOCK180 are cytosolic in quiescent cells. Upon FcyR engagement both of these GEFs are recruited to phosphorylated tyrosines formed by the signalling complex, through their SH2 domains (Lee *et al.* 2007a, Patel *et al.* 2002). We therefore considered whether tyrosine phosphorylation controls the detachment of the GEFs from the cup that ultimately leads to actin clearance, enabling completion of phagocytosis. Phosphotyrosine-specific immunostaining of cells performing frustrated phagocytosis was used initially to analyse this possibility. As shown in Figure 4.4A-A’, ligation of FcyR induced localised tyrosine phosphorylation, as known to occur during phagocytosis of particulate material (Cooney *et al.* 2001). As phagocytosis progressed, the advancing edge of the pseudopods became rich in phosphotyrosine but, remarkably, the central part of contact area became gradually dephosphorylated (Figure 4.4B, B’). This region is equivalent to the base of the phagocytic cup, which in parallel experiments was shown to become similarly dephosphorylated at a comparable stage of pseudopod extension (not shown). To quantify the depletion of phosphotyrosine in cells undergoing frustrated phagocytosis, a region of interest in the middle of the cell was divided by a region at the leading edge to provide a “clearance index” (see Methods for details). This approach confirmed the statistical significance of the localised dephosphorylation (Figure 4.4C). Interestingly, when a similar calculation was applied to the density of F-actin, an almost identical clearance index was estimated (Figure 4.4C), suggesting a correlation between these events.

**Role of tyrosine phosphatases in actin clearance:** The net dephosphorylation observed at the base of the phagocytic cup could be due to either reduced kinase or increased phosphatase activity. Src-family kinases are the primary source of tyrosine phosphorylation during FcγR-initiated phagocytosis (Cooney *et al.* 2001). We analysed the distribution of Lyn, one of the principal Src kinases engaged, during phagocytosis. As expected, active Lyn tagged with GFP was found to associate constitutively with the plasmalemma. During the course of phagocytosis
Figure 4.4: Dynamics of phosphotyrosine accumulation during frustrated phagocytosis
RAW cells stably transfected with GFP-actin were suspended and allowed to settle onto IgG-coated coverslips. After 2 min (A,A’; early stage) or 5 min (B,B’; late stage) the cells were fixed and immunostained for phosphotyrosine. (C) Actin and phosphotyrosine density were quantified at the base of cells like those in B and a “clearance index” calculated, as described in Methods. Data are means ± SE of at least 20 determinations from 6 separate experiments. Scale bar = 10 μm.
Figure 4.4
partial clearance of Lyn-GFP from the center of the contact region was observed (Figure 4.5A-B) an effect that was more noticeable at advanced stages of phagocytosis (not shown). These observations are consistent with earlier findings, where moderate clearance of a catalytically-inactive form of Lyn was attributed to focal insertion of endomembranes by exocytosis (Lee et al. 2007b).

While reproducible, the partial depletion of Lyn (and presumably other Src kinases) is modest (Figure 4.5C) and considerably smaller than the reduction in phosphotyrosine and actin. We therefore considered the involvement of phosphotyrosine phosphatases. Because not all the phosphatases active during phagocytosis have been identified, initially we broadly inhibited phosphatases using peroxide derivatives of vanadate (Trudel et al. 1991), hereafter called pervanadate. A brief pretreatment with pervanadate greatly increased the accumulation of phosphotyrosine when the cells adhered to IgG-coated coverslips (cf. Figure 4.5D and E). Of note, though phosphotyrosine accumulation was still greatest at the edges of the adherent surface, a large amount persisted in the center, comparable to or greater than that found at the edges of an untreated cell (Figure 4.5F). The inhibition of tyrosine dephosphorylation altered actin dynamics during phagocytosis. As also shown in Figure 4.5, actin failed to clear from the center of the frustrated cup, mimicking the phosphotyrosine pattern. Cell spreading was curtailed by pervanadate, possibly by limiting the available actin or actin-associated proteins at the advancing edges.

**Recruitment of phosphatases is phosphatidylinositol-3 kinase dependent:** Though the full complement of tyrosine phosphatases active during phagocytosis is not known, the SH2 domain-containing protein phosphatase (SHP) is thought to play an important role (Ganesan et al. 2003). We attempted to study the distribution and dynamics of SHP during phagocytosis, but neither available antibodies nor GFP-tagged constructs proved to be satisfactory. Instead, we chose to analyse the distribution of Gab2. This adaptor protein mediates the recruitment of SHP to the FcγR signalling complex (Gu et al. 2003). In accordance with earlier findings, we found that Gab2 concentrates markedly at sites of phagocytosis (Figure 4.6A,C). Association of its PH domain with PtdIns(3,4,5)P3 is a key determinant of Gab2 translocation to the membrane (Gu et al. 2003). This was confirmed by treating cells with the phosphatidylinositol 3-kinase (PI3K)
Figure 4.5: Distribution of tyrosine kinases and phosphatases during phagocytosis

RAW cells were transiently transfected with GFP-Lyn and either challenged with 8.3 μm IgG-opsonised beads (A) or sedimented onto an IgG-coated coverslip (B) and representative images acquired after 3 and 5 min, respectively. A typical line scan of the fluorescence intensity of GFP-Lyn from a cell spread as in (B) is shown in (C). Phosphotyrosine immunofluorescence (D,E) and GFP-actin distribution (G,H) of cells that were allowed to spread onto IgG-coated coverslips but were otherwise untreated (D,G) or were treated with pervanadate for 30 seconds before attachment to the surface. Typical line scans for control (black) and pervanadate-treated cells (light gray) are shown in (F,I). Scale bars = 10 μm.
Figure 4.5

**Figure 4.5**

- **A** and **D**: Lyn-GFP
- **B** and **E**: Beads
- **C** and **F**: Control
- **G** and **H**: Frustrated

**Legend**

- Lyn-GFP
- Beads
- Control
- Frustrated

**Description**

- **A**: Lyn-GFP in the presence of Beads.
- **B**: Lyn-GFP in the presence of Frustrated conditions.
- **C**: Control experiment showing the baseline activity.
- **D**: Control with Phosphotyrosine staining.
- **E**: Frustrated condition with Phosphotyrosine staining.
- **F**: Control experiment with Phosphotyrosine staining.
- **G**: Frustrated condition with Actin-GFP staining.
- **H**: Control experiment with Actin-GFP staining.
Figure 4.6: Actin clearance is phosphatidylinositol 3-kinase dependent

RAW cells were transiently transfected with GFP-Gab2 and either left untreated (A) or treated with 100 nM wortmannin (WM; B) for 20 min before exposure to 8.3 \( \mu \text{m} \) IgG-opsonised beads. In (C) the fluorescence intensity per pixel of GFP-Gab2 at the cup was compared to that in the cytosol. Data are means ± SE of at least 60 similar intensity ratio determinations. Distribution of phosphotyrosine (pY; panel D) or Lyn-GFP (panel E) in cells pretreated with wortmannin and allowed to settle onto an IgG-coated surface. An x vs. y view of a confocal slice at the base (top) and an x vs. z reconstruction (bottom) are illustrated. Typical line scans of the fluorescence intensity of pY (F) or GFP-Lyn (G) from control (black) or wortmannin-treated cells (light gray). RAW cells stably transfected with GFP-actin were treated with wortmannin and then exposed to 8.3 \( \mu \text{m} \) IgG-opsonised beads (H) or suspended and allowed to settle onto IgG-coated coverslips (I). Representative images were acquired 5 min after engagement of Fc\( \gamma \) receptors. (J) Effect of wortmannin on phagocytic efficiency. The phagocytic index is expressed as beads internalised per cell. Data are means ± SE of at least 6 experiments, each counting at least 50 cells. All scale bars = 10 \( \mu \text{m} \).
Figure 4.6

Control Wortmannin

Gab2-GFP

A B

pY + Wortmannin Lyn + Wortmannin

D E

XY

F G

XZ

H I

Beads + Wortmannin Frustrated + Wortmannin

J

Phagocytic Index

0.0 0.5 1.0 1.5 2.0 2.5

CTRL WM

Cup to Cell Ratio

Control Wortmannin

1 2 3

* *
inhibitor, wortmannin. As reported, Gab2 recruitment to the phagocytic cup was abrogated by inhibition of PI3K (Figure 4.6B,C).

If Gab2 mediates the recruitment of SHP and this phosphatase contributes to dephosphorylation during phagocytosis, inhibition of PI3K is predicted to enhance and prolong the accumulation of phosphotyrosine at the cup. This prediction was tested experimentally in Figure 4.6D. The PI3K inhibitor did not affect the initial interaction between the cells and the opsonised surface, but limited the degree of spreading, consistent with the reported ability of wortmannin-treated cells to engulf small (≤3 μm) particles, but not larger ones (Araki et al. 1996). When compared to control cells at a similar stage of spreading, wortmannin-treated macrophages attained a higher density of phosphotyrosine (Figure 4.6D,F). Similar results were obtained using the chemically unrelated PI3K inhibitor LY294002 (not shown). The increased tyrosine phosphorylation was most likely caused by diminished phosphatase, rather than elevated kinase activity. This is suggested by the observation that Lyn was not significantly accumulated at the cup in cells treated with wortmannin (Figure 4.6E,G).

**PI3K inhibition prevents actin clearance:** Inhibition of PI3K qualitatively resembled the effect of pervanadate on tyrosine phosphorylation. Both treatments also limited the extent of pseudopod spreading. Because pervanadate prevented clearance of actin from the base of the cup, we analysed whether wortmannin or LY294002 produced the same effect. As reported earlier actin does polymerise at sites of phagocytosis in cells devoid of PtdIns(3,4,5)P₃ (Figure 4.6H,I). However, pseudopod progression is halted at a comparatively early stage, consistent with previous observations (Araki et al. 1996). Importantly, actin remained polymerised at the base of the phagosome for extended periods of time (≥10 min), long after depolymerisation is apparent at the site of initial contact in control cells. Thus, PI3K activity is required for actin clearance. The inability of actin and its associated proteins to turn over and relocate to the advancing edge of the phagocytic cup can explain the inhibitory effect of wortmannin or LY294002 on phagocytosis (Figure 4.6J).

### 4.4 Discussion

The salient observation reported in this study is that, following active polymerisation, actin filaments undergo dynamic disassembly during the course of phagosome formation, a
phenomenon that is more readily apparent, and likely more important, during the ingestion of larger particles. The disassembly is first noticeable in the region where the receptors are initially engaged, i.e. at the base of the phagocytic cup, where actin is initially polymerised. This sequence is repeated centrifugally as the pseudopods advance and new receptors become engaged. This pattern suggests that receptor activation triggers a timing mechanism whereby polymerisation is initiated rapidly and disassembly develops more slowly. We propose that the actin disassembly process, but not its initial polymerisation, involves the activation of PI3K.

Several possible functions were suggested in the Introduction for the active clearance of actin from the base of the phagocytic cup. One of these is to reduce mechanical strain, enabling the membrane to curve around the particle. Careful perusal of the literature and of our own data, however, suggests that this may not be an important factor. Curvature is greater in the case of small particles (e.g. 1-2 μm diameter), yet these can be engulfed in the presence of wortmannin or LY294002, conditions that preclude actin disassembly. Instead, we feel that recycling of actin or of determinants of its assembly to advancing sites of active polymerisation is the main purpose of the dissociation process. Without this step the availability of critical factors may be compromised, limiting the ability of pseudopods to extend beyond a defined length. This proposed mechanism can explain the perplexing observation that phagocytosis of large, but not of small particles is inhibited by inhibitors of PI3K (Araki et al. 1996, Beemiller et al. 2010).

What are the possible mechanisms whereby PtdIns(3,4,5)P3 or other PI3K products promote the depolymerisation of actin? Three separate events were identified in this study that likely contribute to the termination of Rac/Cdc42 activity at sites of phagocytosis: i) a partial decrease in the density of Lyn and likely other Src kinases at the base of the phagocytic cup, ii) the release of Tiam1 from the phagocytic cup and iii) the dephosphorylation of phosphotyrosines that recruit Rho-family GEFs. As discussed elsewhere (Lee et al. 2007b) the partial disappearance of Lyn is attributable to dilution by incoming membranes, delivered by focal exocytosis. This process is likely to be PI3K-dependent since the increase in surface area that accompanies phagocytosis is inhibited by wortmannin (Zhang et al. 1998). The release of Tiam1 from the membrane is also affected by the generation of PtdIns(3,4,5)P3. We found that this GEF attaches to the membrane via association of its PH domain to PtdIns(4,5)P2 and had earlier reported that PtdIns(4,5)P2 disappears from the base of the cup during phagosome formation (Botelho et al. 2000). Disappearance of PtdIns(4,5)P2 is attributable in part to conversion to PtdIns(3,4,5)P3, but
primarily to hydrolysis by phospholipase Cγ. The activation of this phospholipase and hence the disappearance of PtdIns(4,5)P₂ are stimulated by PtdIns(3,4,5)P₃ (Fukami, 2002). Accordingly, the disappearance of PtdIns(4,5)P₂ from the base of the cup was inhibited by wortmannin or LY294002 (see Figure 3.10). It is difficult to determine unambiguously whether Tiam1 is stimulated during phagocytosis, but its presence at the membrane during the early stages of FcγR engagement make it a candidate contributor to actin polymerisation, and its dissociation upon PtdIns(4,5)P₂ hydrolysis could account for part of the actin disassembly observed. Of note, the drop in surface charge that accompanies PtdIns(4,5)P₂ hydrolysis can by itself accelerate the termination of Rac activity, since this GTPase is retained at the membrane in part by an electrostatic interaction between a polycationic region in its hypervariable domain and the negative charge of the membrane, conferred primarily by PtdIns(4,5)P₂ (Yeung et al. 2006).

Tyrosine phosphorylation is essential for the recruitment and activation of Rac/Cdc42 GEFs at sites of phagocytosis. Conversely, phosphotyrosine dephosphorylation is expected to terminate Rac/Cdc42 activation and, in the face of continuous depolymerizing reactions, produce net actin dissociation. It is therefore noteworthy that inhibition of PI3K was accompanied by a marked reduction in the rate of phosphotyrosine dephosphorylation at the cup, which in turn resulted in retention of actin. Recruitment of SHP via Gab2, a PtdIns(3,4,5)P₃-dependent adaptor, is likely to play a role in the dephosphorylation and actin disassembly, but other phosphatases including the Low Molecular Weight Protein Tyrosine Phosphatase (LMW-PTP) may also contribute (Mancini et al. 2007). Lastly, Rac/Cdc42 activity may be terminated by stimulation of cognate GTPase-activating proteins (GAPs) that may in turn be under the control of PI3K. Indeed, some Rho-family GAPs are endowed with PH domains that can bind PtdIns(3,4,5)P₃. Therefore, generation of the inositide at the cup could recruit and activate GAP activity that would promote actin disassembly. However, we found that not only wild-type Rac but also constitutively-active Rac was cleared from the phagocytic cup (not illustrated). Because constitutively-active Rac is insensitive to GAPs, other factors must have contributed to its detachment and termination of its action at the forming phagosome. Because of their multiplicity and promiscuity, detailed analysis of Rho-family GAPs was outside the scope of this paper, but these proteins should become the focus of future studies.

While clearly able to interfere with actin remodelling, PtdIns(3,4,5)P₃ signals through a variety of additional pathways and may interrupt phagocytosis by other means. For instance, delivery of
endomembranes to provide extra surface area at sites of phagocytosis also appears to be influenced by products of PI3K. While the effects of the kinase are unquestionably complex, we feel that its contribution to actin disassembly is key to the completion of (large) phagosome formation. The finding that the inhibitory effects of PI3K inhibitors are mimicked by jasplakinolide, which targets actin specifically, supports this conclusion.

4.5 Materials and Methods

Reagents and Antibodies: Cy3 and Cy5-conjugated anti-human IgG were purchased from Jackson ImmunoResearch Laboratories. Paraformaldehyde was from Electron Microscopy Sciences. The 4g10 anti-phosphotyrosine primary was from Upstate Biotechnology. Unconjugated 8.31µm polystyrene beads were provided by Bang’s Laboratories Inc. Conjugated phalloidins were from Molecular Probes. Jasplakinolide and wortmannin from Calbiochem. Human IgG, ionomycin, LY294002 and all other reagents from Sigma-Aldrich.

Plasmids: The construction and usage of Gab2-GFP has been described previously (Gu et al. 2003). The PAK1-PBD domain conjugated to YFP was a gift of G. Bokoch (The Scripps Research Institute, La Jolla, CA). cDNA for actin labelled with either eGFP or monomeric cherry was a kind gift of D. Knecht (University of Connecticut, CT). The calponin homology domain of utrophin (Utr-CH) was a gift of W. Bement (University of Wisconsin, WI), its construction is described in (Burkel et al. 2007). The use of the GFP-DOCK180 fusion was published previously (Lee et al. 2007a) and was generously provided by M. Matsuda (Osaka University, Japan). The c-terminally GFP tagged Vav1 construct was a gift of W. Swat (Washington University School of Medicine, St Louis, MO). Tiam1-GFP encodes the n-terminally truncated C1199 Tiam1 constructed with GFP and was provided by P. Downes (University of Dundee, Scotland). The mRFP version of the PH domain from PLC-δ was constructed as detailed previously (Yeung et al. 2006). Usage and construction of a plasmid expressing the Lyn isoform B fused to GFP has been previously described (Hess et al. 2003).

Cell culture and Transfection: RAW 264.7 cells were obtained from the American Type Culture Collection (ATCC), and were maintained in DMEM supplemented with 5% fetal bovine serum (Wisent) at 37°C and 5% CO2. For transfection RAW cells were grown to 50–60% confluence on glass coverslips and treated with the plasmids plus FuGene HD (Roche Diagnostics) according to the manufacturer's instructions. All cells were used 16-32h after
transfection. RAW cells stably expressing eGFP-actin or mCherry-actin were a kind gift of D. Knecht (University of Connecticut, CT).

**Models of phagocytosis:** 8.31μm polystyrene microspheres with 2% DVB (Bang’s Laboratories) or ethanol washed glass coverslips (see below) were exposed to 1mg/mL human IgG at room temperature for 1h. Both were washed thoroughly in PBS before usage. For bead experiments, cells were exposed to opsonised particles for 3, 7 or 10 minutes (early, middle or late time points respectively) and then fixed in 4% PFA for 20 minutes at 4ºC. Cells were kept under PBS and used within 2 days of fixation. For calculation of phagocytic indices, opsonised particles were prelabelled with a low concentration of fluorescently labelled secondary antibody before addition to the cells. Phagocytosis was allowed to continue for 20 minutes before fixation, whereupon a second secondary antibody was used to label external beads.

Frustrated phagocytosis has been described previously (Marshall *et al.* 2001). Briefly, RAW cells grown on plastic culture dishes were scraped using a rubber policeman and resuspended in HPMI (Wisent). Cells were added to prewarmed coverslips and allowed to ‘spread’ while being imaged live or fixed after 3 or 8 minutes for early and late time points respectively.

**Pharmacological treatment:** To stabilise actin, beads were added to the cells for 3 minutes before the addition of 1μM jasplakinolide and were then allowed to complete the assay as described above. Phosphatidylinositol-3 kinases were inhibited by pretreating the cells for 20 minutes with 100nM wortmannin or 100μM LY294002 which were found to have the same effect for our experiments (data not shown). Cells co-transfected with Tiam1-GFP and PLCδ PH mRFP were treated for 10 minutes with 10μM ionomycin to deplete plasma membrane phosphatidylinositols (4,5)-bisphosphate. Production of the peroxide derivatives of orthovanadate has been detailed previously (Trudel *et al.* 1991).

**Immunofluorescence:** Cells having undergone frustrated phagocytosis (see above) were fixed in 4% PFA for 20 minutes at 4ºC and washed two times with PBS. Cells were blocked and permeabilised in 5% donkey serum, 0.1% Triton X-100 in PBS for 60 minutes. Primary antibody against phosphotyrosine (clone 4G10, Upstate Biotechnology) was added overnight at 4ºC, in PBS with 1% BSA. Cells were washed with PBS and a Cy3 labelled secondary antibody (Jackson ImmunoResearch Laboratories) in PBS with 1% BSA for 60 minutes, before being
further washed with PBS. Where required, fluorescently labelled phalloidin was used to label actin in fixed cells as directed by the manufacturer.

**Microscopy:** Bead phagocytosis and frustrated phagocytosis were both imaged using Nipkow spinning disk confocal microscopy. A Yokagawa spinning disk head was mounted on a Zeiss Axiovert 200M inverted microscope. Samples were illuminated using 488nm, 561nm or 605nm laser light (Spectral Applied Research) as appropriate, with light passing through thin layer filters as necessary (Semrock). Images were captured on a Hamamatsu C9100-13 ImagEM camera driven with the Volocity software (Improvision). Live frustrated phagocytosis was also captured using Total Internal Reflection Fluorescence Microscopy (TIR-FM), on a Zeiss Axiovert 200 microscope with 488nm or 561nm lasers as required. Camera and acquisition setup as above.

**Quantitation and repetition:** Phagocytic indices were calculated by exposing RAW cells to opsonised and fluorescently labelled polystyrene particles for 20 minutes then fixing and labelling external beads. The index is calculated as the average number of beads per cell in randomly selected fields. Clearance index of cells undergoing frustrated phagocytosis were calculated as follows: The outermost boundary of the cell as imaged with TIR-FM was selected and the area between this line and the same line shrunk towards the centroid by 5 microns was considered region 1 (R1). A second region (R2) was calculated by creating a 5 micron circular region at the centroid of the cell. After subtraction of the average background signal, the ratio (R2/R1) provides an indication of clearance at the middle of the ‘cup’, whereby a value of 1 denotes no clearance. All image analysis was undertaken using ImageJ (National Institutes of Health).

All results were confirmed by repetition in at least 4 experiments on different days. All images and any single cell data (such as line scans) are typical examples of the results. Where deemed necessary for clarity, image histograms were altered in such a way as not to interpolate or lose any data. Error bars represent standard error of the mean.
4.6 References


4.7 Supplementary Figures

Figure 4.7: Actin clearance is evident regardless of actin probe used.

(A) RAW cells were transiently transfected with an mCherry labelled calponin homology domain of utrophin (Utr-CH) and subsequently challenged with 8.31μm opsonised beads for 3 (Early), 5 (Mid) or 8 (Late) minutes before fixation and imaging. (B) Alternatively, RAW cells were exposed to particles for the same time points before fixation and stained with fluorescently labelled phalloidin to label filamentous actin. Scale bar represents 10 microns. Asterisks denote the position of a bead as confirmed by DIC imaging (not shown).
Figure 4.7
**Figure 4.8: P-Rex1 is not recruited to forming or internalised phagosomes.**

RAW cells were transiently transfected with GFP-P-Rex1 and challenged with opsonised 8.3 μm beads. Cells were imaged live from cup formation (A) to internalisation (B). Asterisks denote the position of a bead as confirmed by DIC imaging (not shown).
Figure 4.8
5  Thesis Discussion and Future Directions

The main chapters of this thesis have described novel findings in the field of Microbiology and Cell Biology specifically related to host-pathogen interactions. Any single piece of work however, is not exhaustive. As such, the following sections will comment on recent published findings in relation to the projects, as well as further exploring concepts touched upon in the main body of work.

5.1  Protein Domains as Biomarkers for Lipids

5.1.1  Functional Roles of Lipids

The nature of the cellular lipid membrane has been a topic of great interest for many years. The suggestion of a fluid mosaic model suggested a heterogeneous milieu of transmembrane proteins freely diffusing in a ‘sea’ of lipids (Singer & Nicolson, 1972). For a long time the lipids were thought simply to act as a barrier and solvent for these proteins. In the last four decades, there has been an increased appreciation for the active role that lipids play in cellular signalling.

Phospholipids have been intensively studied in the context of signalling, specifically phosphatidylinositol (PI) and its derivatives. PI is an amphiphilic lipid, having a glycerol backbone with two acyl chains (usually stearate and oleate) and a phosphate-linked inositol sugar head-group. The head-group can be phosphorylated in any combination of the 3, 4 or 5 positions, producing seven potential derivatives (see Figure 5.1). Phosphatidylinositol (4,5)-bisphosphate (PtdIns(4,5)P₂) has been the topic of much study, as it directly regulates many proteins, such as the WASP/WAVE proteins described in Section 1.4.6.1. In this case, the charged head-group binds to WASP/WAVE relieving an autoinhibitory interaction and allowing binding to actin nucleating proteins. Many other examples of this type of direct regulation exist, from ion channel regulation (Huang, 2007) to nuclear signalling events and cell cycle regulation (Bunce et al. 2006).

As well as direct regulation, PtdIns(4,5)P₂ can also act as the source of several important second messengers, namely inositol (1,4,5) trisphosphate (IP₃) and diacylglycerol (DAG). These signalling molecules are the product of PtdIns(4,5)P₂ hydrolysis by phospholipase C (PLC). IP₃
Figure 5.1: Metabolism of phosphatidylinositol derivatives

Phosphatidylinositol (PI) can be phosphorylated on any of the 3, 4 or 5 positions. Derivatives may be singly phosphorylated (shown in orange), doubly phosphorylated (shown in blue) or phosphorylated on all three positions giving rise to PtdIns(3,4,5)P₃ (shown in purple). Addition of phosphate groups occurs through the action of PI-kinases (green arrows) whereas dephosphorylation occurs through PI-phosphatases (red arrows). Only pathways described (solid arrow) or proposed (dashed arrow) in the literature, *circa* 2009, are shown.
Figure 5.1
can interact with IP3-receptors in the endoplasmic reticulum to stimulate cytosolic calcium increase. DAG production initiates survival signals generated through protein kinase C (PKC) activation. These pathways will not be further covered here. Nevertheless they illustrate that lipids play an active role beyond serving simply as a barrier and solvent.

5.1.2 Phospholipid-Binding Domains

Proteins interact with lipids through specialised binding domains that are usually modular and very well conserved between proteins. The first characterised lipid-binding domain was the pleckstrin-homology domain of phospholipase C (PLC) delta. This domain binds with reasonable affinity and specificity to PtdIns(4,5)P2 and serves to localise PLC to its substrate at the plasma membrane. Since then, many lipid-binding protein domains have been identified, each with varying specificity and affinity for target lipids (see Table 3). When isolated and conjugated to fluorescent proteins, these domains are capable of reporting the localisation of phospholipids in the cell. This technique has been successfully used in the three main chapters of this thesis, to report the dynamic localisation, production and degradation of phospholipids in the cell (for examples see Figure 2.2, Figure 3.10, and Figure 4.3).

5.1.3 Benefits, Detriments and Alternatives

The largest benefit of these phospholipid probes is the ability to use them in live cells. With the advent of fluorescent protein technology, these modular domains can be labelled with proteins bearing almost any spectral properties. This makes them highly advantageous for double or triple colour labelling and thus for colocalisation studies. As more protein domains are isolated, researchers also have reasonably selective probes for lipids. As an example, one of the earlier isolated domains, the PH domain from Akt, has been used for labelling PtdIns(3,4,5)P3 in cells, however it was also shown to bind to PtdIns(3,4)P2. A more recently discovered probe, the Gab1 PH domain, is more selective for PtdIns(3,4,5)P3 and as such is becoming the tool of choice for studying this lipid.

Another property of these modular domains is that they have characteristic rates of association and dissociation with their target lipid. When not bound, the lipids are free to interact with endogenous effectors and regulatory proteins. Care must be taken when selecting and engineering these probes as, if the affinity of the isolated probe is too low, more protein will be
Table 3: Modular interaction domains used to visualise lipids

Many proteins use modular lipid-binding domains such as PH or C1 domains to interact with lipids. Using techniques in molecular biology, the sequences for these domains can be conjugated to sequences encoding fluorescent proteins. When the resultant cDNA is expressed in cells the now fluorescent protein domains can be used to spatially and temporally localise lipids in a live cell.
Table 3: Modular interaction domains used to visualise lipids

<table>
<thead>
<tr>
<th>Lipid</th>
<th>Domain (protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diacylglycerol</td>
<td>C1 (PKC)</td>
</tr>
<tr>
<td>Phosphatidylserine</td>
<td>C2 (lactadherin)</td>
</tr>
<tr>
<td>PtdIns(3)P</td>
<td>FYVE (EEA1), PX (P40, NADPH oxidase)</td>
</tr>
<tr>
<td>PtdIns(4)P</td>
<td>PH (OSBP2), PH (FAPP1)</td>
</tr>
<tr>
<td>PtdIns(3,4)P₂</td>
<td>PH (Akt), PH (TAPP1)</td>
</tr>
<tr>
<td>PtdIns(4,5)P₂</td>
<td>PH (PLC-delta)</td>
</tr>
<tr>
<td>PtdIns(3,4,5)P₃</td>
<td>PH (Akt), PH (Gab1)</td>
</tr>
</tbody>
</table>
cytosolic and so the background fluorescent signal will be undesirably high. Alternatively if the affinity of the domain for its lipid is too high, the lipid can be tightly bound by the domain, preventing interaction with endogenous effectors and potentially perturbing native signalling.

These properties can actually be engineered to the advantage of the researcher. A low affinity lipid binding domain, the Fab 1, YOTB, Vac 1, EEA1 (FYVE) domain, weakly binds PtdIns(3)P when expressed alone. Under these circumstances it is of limited use for localisation studies due to prohibitively high background signal. Engineering multiple FYVE domains increases the avidity of the probe for the lipid, without compromising native signalling (Kelley & Schorey, 2004). Under some circumstances perturbation of native signalling can be a desirable effect; indeed this method was implemented in Chapter 2 to inhibit PtdIns(4,5)P₂ signalling. In this case, the PH domain of PLCδ was expressed in tandem form. This high-avidity probe sequesters PtdIns(4,5)P₂ in the plasma membrane, masking it and effectively blocking PtdIns(4,5)P₂-mediated regulation of proteins and the production of important second messengers (see Figure 2.5 and accompanying text).

Despite their benefits, there are disadvantages to using these modular domains. These are mostly side-effects of the rapid association and dissociation of the probe with its target. As previously mentioned, domains are usually chosen so as not to perturb endogenous signalling. For this reason, it is especially important not to over-express the probes and to perform controls where possible. Another disadvantage of the on/off rates of these probes is that background signal (from dissociated probe) is in most cases, an inevitability. In order to limit the impact of this, confocal or other thin focal plane microscopy is preferable to the relatively thick focal depth of epifluorescence microscopy.

A final problem with having a probe actively dissociating, is that much of the useful data for lipids relies upon having good measurements of lateral mobility. When using lipid-binding domains, these measurements rely upon the rate of dissociation of the probe being negligible when compared with the lateral diffusion of the lipid being studied. Unfortunately this is typically not the case and alternative means are required for studying the lateral diffusion of lipids (see Section 5.1.4 below).
Alternatives to lipid-binding domains do exist for labelling lipids in the plasma membrane. Perhaps the most intuitive, is the use of the readily available fluorescently-labelled lipids. Small chemical fluorophores such as Bodipy or rhodamine can be conjugated to either the acyl tails or head-group of lipids. These lipids can themselves be conjugated to a carrier such as albumin or resuspended in chloroform and loaded onto the cells. Typically they will then insert into the plasma membrane (Golebiewska et al. 2008).

This technique is not without its own problems. The choice of labelling position is a difficult one that must be considered with care. Label the tail and you will likely disrupt packing and the hydrophobic-interaction with other lipids. If the head-group is modified you are technically no longer looking at the same lipid and so conclusions based upon lipid-lipid or lipid-protein interactions may not hold; modifying the lipid in this way can prevent interaction of the lipid with binding partners. These interactions may dictate the kinetic properties of endogenous lipids. Finally, loading exogenous lipids into cells can itself can have undesirable effects. Blebbing or ruffling can occur as the cell compensates for changes in surface area and shape.

5.1.4 Quantitative Measurements of Lipids Dynamics

Despite the problems described in the previous section, it is important to quantitatively study the diffusion of lipids in the membranes of cells. Much can be learnt from studying lipids in situ. Once rates of diffusion are calculated, statistical dwell times and probabilities for protein or lipid interaction can be calculated. All of this is valuable information when trying to understand proteins such as receptors that exist and interact with the surrounding lipid milieu in a temporally and spatially complex manner. Several research groups have recently been able to calculate some of these parameters using innovative techniques that will be discussed below.

5.1.4.1 Fluorescence Correlation Spectroscopy (FCS) and Fluorescently-Labelled Lipids

Two main methods can be used when attempting to study the mobility of lipids in the plasma membrane. Single molecule spectroscopy can be used to calculate the parameters of single probes; however these data usually have a low signal to noise ratio due to the stochastic nature of single molecules. The alternative is to use bulk or averaged measurements in order to discern the properties of a population. One technique for the latter method is Fluorescence Correlation Spectroscopy (FCS). The reader is directed to a review on the technique for more details (Kim et
al. 2007) but briefly, a small focal volume is illuminated and the emission intensities of fluorescently labelled probes are measured as they diffuse in-to and out-of the excitation volume. Many fluorophores are present in the focal volume at any time and, as such, the mean residency time of fluorophores and thereby, the diffusion coefficient of the probe, can be calculated.

This technique has been applied to soluble probes as well as membrane anchored proteins in the past, however the problems described in Section 5.1.3 above are still present. Using protein-based lipid-binding domains may still principally report the on/off rates of the proteins and not the diffusion rate of the lipid itself.

Recently, Golebiewiewska et al. microinjected fluorescently labelled PtdIns(4,5)P₂ micelles directly into cells and used FCS to calculate an in situ diffusion coefficient (Golebiewska et al. 2008).

Upon incorporation into the inner leaflet of the plasma membrane, FCS measurements calculated the diffusion coefficient of PtdIns(4,5)P₂ to be 0.8 μm/s². This technique also allowed comparison of PtdIns(4,5)P₂ diffusion on the inner and outer leaflet, as well as on membrane blebs. Interestingly, PtdIns(4,5)P₂ on the inner leaflet diffused threefold more slowly than PtdIns(4,5)P₂ in blebs. The authors conjecture that electrostatic interactions with the basic residues of inner leaflet-associated proteins are most likely to account for this reduced diffusion (Golebiewska et al. 2008), which is highly suggestive of a pool of PtdIns(4,5)P₂ being constitutively bound, but released in response to specific signals. This theory has been suggested in the past as an explanation as to how PtdIns(4,5)P₂ can regulate so many different processes at the plasma membrane (Janmey & Lindberg, 2004, Aikawa & Martin, 2003).

Two main problems exist with this system. Firstly, labelling lipids even with a small chemical dye is a compromise. Fluorescently labelling the acyl chains of lipids can alter lipid-lipid interactions in the membrane and thus potentially alter interactions that can have an effect on the reported diffusion coefficient. Labelling the head-group alters the ability of a lipid to interact with head-group interacting proteins, which may also alter dwell times or interactions. Given the suggestion that the diffusion of PtdIns(4,5)P₂ may be regulated by head-group-binding proteins it seems beneficial to label the tail groups as did Golebiewiewska et al. It is important however, to consider that the results always come with the above-mentioned caveats.
The second problem in working with labelled exogenous lipids relates to lipid recycling and enzymatic activity. At 37°C, lipid-modifying enzymes are continually metabolising lipids some of which are recycled through constitutive endocytosis. Golebiewiewska et al. minimised these problems by conducting experiments at 25°C. While this reduces both metabolism and endocytosis, decreasing temperature may have undesirable effects on the fluidity of the plasma membrane. Reducing temperature moves the lipid structure away from a lipid disordered phase and towards a more crystalline phase. Whether this will occur significantly with a 37°C to 25°C shift was not addressed, however it is an important consideration that can impact diffusional measurements.

Further experiments utilising this and other similar techniques should aid in understanding the lipid composition and dynamics at the plasma membrane. As this is a bulk measurement, discerning separate ‘pools’ of PtdIns(4,5)P2 in the plasma membrane may not be possible, but this technique, despite being technically challenging, is certainly applicable to the study of other lipids.

5.1.4.2 Fluorescence Recovery After Photobleaching (FRAP) with Lipid-Binding Domains

Fluorescence Recovery After Photobleaching (FRAP) is a powerful technique for calculating diffusion rates of fluorescently-labelled proteins in the plasma membrane (see Chapter 3 for more details). As previously described however, labelling lipids and having them report physical parameters accurately is not a trivial procedure. Instead, Hammond et al. used FRAP techniques to investigate the kinetics of phospholipids in terms of the lateral diffusion and dissociation of lipid binding domains (Hammond et al. 2009).

In conventional FRAP experiments using membrane-anchored fluorescent proteins, the recovery of fluorescence into a bleached region is purely the result of lateral diffusion (Axelrod et al. 1976). When using lipid-binding domains for FRAP, recovery of fluorescence can be the result of lateral diffusion of probe that remains bound to lipid or alternatively, the result of exchange of the photobleached probe with fluorescently labelled cytosolic probe.

The key difference between these two modes of recovery is the spatial distribution of the probe as the bleached region is recovering. Given the nature of the photobleaching laser, the bleach
region has a Gaussian profile of radius (r) and depth (d) (see Figure 5.2B&C). If recovery is purely the result of lateral diffusion, the profile will widen and become more shallow as photobleached probe diffuses laterally out of the bleach region and surrounding unbleached probe diffuses in (see Figure 5.2B). Importantly, because there is no cytosolic exchange in this model, the area under the recovery profile remains constant but is spatially redistributed. If however, recovery is purely the result of photobleached probe dissociating from the lipid and being exchanged for unbleached probe in the cytosol, the radius (r) of the bleach profile would remain constant and the depth (d) would decrease (see Figure 5.2C).

In reality, using soluble fluorescent lipid-binding probes, the recovery is a combination of both models, with some of the recovery being the result of lateral diffusion and some being the result of exchange. As these two modes of recovery affect the recovery profile differently (as described above), the two factors can be deconvolved, yielding both a lateral diffusion coefficient and a membrane dissociation time.

Applying this technique to the study of membrane PtdIns(4,5)P₂, Hammond et al. used the PH domain of PLCδ for FRAP analysis. They found that in an unstimulated cell, the effective lateral diffusion of PtdIns(4,5)P₂ was 1.24 μm/s², a figure in good accordance with the value derived from FCS measurements (see previous section or Golebiewiewska et al. 2008). Furthermore the membrane dissociation time constant was found to be 2.44 seconds. This suggests that while lateral diffusion is free to occur when these probes are bound to lipids, the distance they will diffuse is limited to several microns. Given the fast dissociation of probes, this helps to explain how the cell can respond very quickly to the localised production of phosphatidylinositol lipids. Relying upon lateral diffusion to transit effectors across a cell tens of microns in diameter would be prohibitively slow.

In theory this technique can be applied to any protein with lipid interaction domains. Interestingly there is no reason why the same technique could not be applied to protein-protein interactions. Many signalling pathways including the ones outlined in this thesis, rely upon modular protein-protein interaction motifs such as SH2 domains. Elucidating the temporal and spatial recruitment of these domains to the upstream receptors would greatly aid in our understanding of the kinetics of signalling.
Figure 5.2: Schematic representation of FRAP recovery profiles

(A) In Fluorescent Recovery After Photobleaching (FRAP) experiments, a cell expressing a fluorescent probe has a region of fluorescence irreversibly photobleached. When the intensity of the bleach region is measured over time, the rate of fluorescence recovery can be used to calculate the diffusion characteristics of the probe. (B) If the recovery is purely the result of lateral diffusion of the probe, the recovery profile will become wider but shallower during the course of recovery. This is typical of a membrane anchored probe. (C) If recovery is solely the result of exchange with a cytosolic pool of fluorescent probe the radius of the bleach profile will remain constant, however the depth will decrease with time.
Figure 5.2

A

Pre-bleach  
Post-bleach  
Recovery

Cell

B

Recovery profile:
Pure lateral diffusion

C

Recovery profile:
Pure exchange
In summary, both FCS and FRAP are well-established techniques in the field of cell biology.Outlined above are two examples of how this technology can be applied in innovative ways to answer pertinent questions in the field.

5.2 Activation and Abrogation of Fc-gamma Receptor Mediated Signalling

5.2.1 Clustering and the Initiation of Phagocytosis

Phagocytosis is initiated when receptors are brought into close proximity in a process termed ‘clustering’. It is yet unclear what the nature of clustering is, or why it is required. Initiation of signalling occurs through the Src-family of proteins, which are membrane-anchored kinases. Current thinking suggests that clustering serves to directly immobilise the lipid-anchored Src-kinase through lipid-lipid or lipid-protein interactions. This would increase the dwell times of SFKs, thus increasing the likelihood of receptor phosphorylation.

This topic is explored in Chapter 3 where we showed that the tail of one SFK showed reduced mobility at the phagocytic cup (see Figure 3.2). The nature of this immobilisation is still unclear, being independent of the actin cytoskeleton beneath the phagocytic cup and cholesterol in the plasma membrane. Curiously the activity of the SFK was required for immobilisation of the kinase, ruling out a simple model of increased protein or lipid density at the phagocytic cup, which would be independent of kinase activity.

This is instead highly suggestive of a role for active SFKs in recruiting other adaptors and transmembrane proteins or directly interacting with lipids in the membrane to create a local microdomain that serves to reduce the mobility of other SFKs. Overall this would increase the magnitude of phagocytic signalling and lead to a positive feedback of signalling once initiated.

5.2.2 Signalling Thresholds and Diffusional Barriers

The system described above would act to prevent full initiation of phagocytic signalling unless a certain density of receptors are clustered. This is important so that the degree of signalling matches the size of the particle. Without these thresholds, the same signalling would be initiated
regardless of the particle ligand (opsonin) density, which is to say that signalling would be the
same for a monomeric IgG versus an opsonised bacterium.

Similarly, phagocytosis should not be initiated unless there are sufficient resources such as actin
available in the cell to complete the process. Likewise if the forming phagocytic cup is not
correctly formed in close apposition to the particle, later events may not lead to the formation of
a full phagocytic cup and closure of the cup would fail. It is therefore important for the
phagocyte to have some way to ‘monitor’ the state of the phagocytic cup and only proceed if
certain criteria are met.

5.2.2.1 Phospholipids as Checkpoints

The idea of a checkpoint for phagocytosis is conceptually a difficult one. The nature of the
checkpoint for the later stages of phagocytosis has recently been suggested to be based on a
barrier function at the forming phagosome, which would act to concentrate and regulate
phospholipids (Swanson, 2009). Many processes throughout phagocytosis rely upon the
presence, formation and interconversion of phosphatidylinositol lipids. PtdIns(4,5)P$_2$ can
regulate the polymerisation of actin as well as active GTPases (Scott et al. 2005). The formation
of PtdIns(3,4,5)P$_3$, however seems to regulate many stages of phagocytic signalling, including
the delivery and fusion of internal vesicles and secondary regulation of GTPases. Indeed, given
the positive feedback associated with the recruitment of the Type I PI3K (Gu et al. 2003), it is a
distinct possibility that the formation of PtdIns(3,4,5)P$_3$ acts as a checkpoint for the completion
of phagocytosis. Interestingly, PI3K inhibition does not prevent the internalisation of small (less
than 3 $\mu$m) particles. This checkpoint is therefore not absolute, as smaller particles may not
require the fusion of internal vesicles to compensate for the amount of plasma membrane that is
internalised with a small phagosome. In this case, the later stages of signalling need not be
initiated.

If phospholipids are acting as a concentration-mediated checkpoint, lipids must be able to
concentrate sufficiently in order to reach a requisite threshold. Furthermore this signalling must
be spatially localised to the region of the phagocytic cup and not elsewhere in the cell. With
respect to PtdIns(3,4,5)P$_3$, after induction of signalling, the Type I PI3K is recruited to the
phagocytic cup through interactions with the adaptor protein Gab2 and through SH2-mediated
interactions with the receptor complex. With the source of PtdIns(3,4,5)P$_3$ localised to the
phagocytic cup, three possibilities will be discussed regarding how the phospholipid itself might be concentrated at the forming cup:

**Metabolic regulation:** In order to form a lipid gradient metabolically, two things are required. Firstly, the localised recruitment of PI3-kinases to the phagocytic cup and secondly, PtdIns(3,4,5)P$_3$ phosphatases or hydrolases are required to be constitutively active everywhere else. This would allow PtdIns(3,4,5)P$_3$ to diffuse out of the cup to be down-regulated, thus maintaining the specificity of the localisation and signalling. Examples of this sort of regulation exist. During chemotactic migration for example, motile cells use spatially localised gradients in order to control the resulting migratory cues.

There is partial evidence in support of this model in phagocytosis, as PI3Ks are recruited specifically to the site of receptor conjugation. This is mediated (as described in Chapter 1) through interactions with the receptor complex and is thus restricted spatially. There are lipid phosphatases that may play a down-regulating role elsewhere in the cell. These would have to be actively excluded from the phagocytic cup or alternatively, would have significantly lower activity than that of the kinase, so as not to preclude accumulation at the cup.

Whilst possible, it is unlikely that this system would be able to maintain a sharp boundary at the edge of a phagocytic cup. A gradient of lipid probe would be expected, strongest at the cup but decreasing away from it. This is not the PtdIns(3,4,5)P$_3$ pattern that is normally seen in phagocytosis (see Figure 3.10 for examples).

**Curvature-induced regulation:** A more recent hypothesis to account for lipid restriction to the phagocytic cup suggested the existence of a barrier to diffusion. This requires no physical barrier *per se*, but relies upon the formation of a region of extreme curvature in the membrane (such as is seen at the tip of the extending pseudopod). Lipids pack into the membrane based upon the size and charge of their head-groups, the length of their acyl tails and also the shape of their fatty acid tails (which in turn is based upon their degree of saturation). Based upon these factors, lipids can be broadly classed into three groups: Cone-shaped lipids have small head-groups and typically bulky, unsaturated acyl chains. Inverted cone-shaped lipids have the opposite structure, typically with saturated single acyl chains with a large or highly charged head-group. Finally, lipids with intermediate head-groups and acyl chains can be considered to have a cylindrical profile.
It is yet unclear whether lipids induce membrane curvature based upon their shape or if they will be sequestered into areas where this curvature already exists. If and when this is further probed, the answer is likely to be a combination of both. Regardless, at the tip of the extending pseudopod, a highly negative curvature exists on the inner leaflet of the membrane and a highly positive curvature on the outer leaflet. It is likely that cone-shaped and inverted cone-shaped lipids will pack into these regions respectively and provide a highly dense and poorly exchangeable lipid environment that could prevent the free diffusion of cylindrical lipids such as the phosphoinositides PtdIns(4,5)P$_2$ and PtdIns(3,4,5)P$_3$.

With such curvature-induced barriers the diffusional limitation would likely be largely exerted through acyl tail interactions. It is believed that up to 50% of the PtdIns(4,5)P$_2$ is bound to proteins of some sort through its head-group (Golebiewska et al. 2008). Any selective barrier based upon head-group interactions would only act on unbound lipid, whereas a tail-mediated barrier function would work regardless of head-group interactions. This makes curvature-induced regulation a favourable choice for a potential barrier; however a good system with which to test this hypothesis has not yet been developed.

**Physical regulation;** Perhaps the most intuitive form of a diffusional barrier is that of a physical barrier. A tight ring of protein either interacting with lipids or inserting into the membrane could satisfy all of the previously described criteria of a diffusional barrier.

Several examples of protein-based diffusional barriers have been demonstrated in other systems. Epithelial cells are an excellent example of polarised cells, having a highly organised apical surface adapted to luminal interaction, and a basal side that can communicate with the underlying organ matrix. Polarity is maintained partly through the tight junctional complex that encircles the cell. This barrier forms a scaffolding for cell-cell interactions and plays an important role in preventing paracellular leakage. Of interest here, the tight junction can also act as a diffusional barrier to lipids on the outer leaflet of the cell membrane (Matter & Balda, 2003, Dragsten et al. 1981).

Neuronal cell types also require rigorous control over cellular domains. Within one cell, the axon must be separated from the dendritic spines to provide polarity to the received and transmitted signals. The axon is separated from the somatodendritic body through a protein ring, where a concentration of actin, ankyrin, voltage-dependent sodium channels and other proteins directly
act to restrict the diffusion of lipids and membrane-associated proteins between the two compartments (Hedstrom et al. 2008). More interesting still is the modulation of the dendritic spines that are responsible for the modulation of post-synaptic action potentials. These nodes on the dendritic extensions are highly plastic and yet when formed, restrict the diffusion of AMPA and other receptors effectively modulating the post-synaptic potential. The main structural protein at the neck of the spines is thought to be a filament forming protein of the septin family (Tada et al. 2007). Interestingly homologs of this protein are also found at the bud neck in yeast (Cid et al. 2001) and the cleavage furrow of mammalian cells (Xie et al. 1999) offering a promising candidate for a physical diffusion barrier.

The three possibilities for a diffusional barrier discussed above each have merits and problems. It is yet unclear which combination of these or other mechanisms are responsible for the diffusional barrier seen during phagocytosis. The physical barrier described above is appealing as it is capable of being selective between the inner and outer leaflets of the plasma membrane. It can also be dynamically regulated and could conceivably act as a barrier to both lipids and membrane-associated proteins as is seen experimentally. The septins are also a likely candidate for this barrier. Inhibition of septin activity has been shown to decrease phagocytic efficiency in macrophages (Huang et al. 2008), however these experiments were conducted with small particles which may not have been sensitive to barrier requirements in the first place.

Overall, further work will be required to elucidate not only the nature of the diffusional barrier, but also the properties with regard to selectivity and dynamic regulation. This is of critical interest in understanding the roles of signalling thresholds in phagocytosis, as well as better understanding the spatial regulation of signalling pathways.

5.2.3 Alternative Mechanisms of Actin Downregulation

Chapter 4 studied the important role that phosphotyrosine-based signalling plays in the clearance of actin at the phagocytic cup. Inhibiting protein phosphatase activity in the cell can prevent the completion of phagocytosis. While the role of phosphatases has been demonstrated, this work does not exclude other means by which signalling can be abrogated, leading to a loss of actin at the phagocytic cup. Some of these alternate mechanisms will be discussed below.
5.2.3.1 Other Protein and Lipid Phosphatases

The importance of phosphatases in the completion of phagocytosis is highlighted by the experiments using pervanadate (see Figure 4.4). This compound irreversibly oxidises the catalytic cysteine that is required for phosphatase activity (Huyer et al. 1997). SHP is thought to be a major protein phosphatase in phagocytosis, however attempts to study this protein directly were unsuccessful. Importantly, this work does not rule out a role for other phosphatases in the clearance of actin from the forming phagocytic cup.

By means of example, one such candidate is the low-molecular-weight protein tyrosine phosphatase (LMW-PTP). This protein shares little sequence homology with other phosphatases, except for the presence of the consensus C-X5-R motif. Furthermore, its mechanism of action has also been shown to be identical to other protein phosphatases (Raugei et al. 2007). The LMW-PTP was originally characterised to play a role in growth factor-mediated remodelling and motility by regulating the GTPase Rho and to some extent Focal Adhesion Kinase (FAK) (Chiarugi et al. 1998). The activity of LMW-PTP is regulated by the phosphorylation state of two essential tyrosines (Tailor et al. 1997). Of interest here is that the second tyrosine is followed by the residues G-N-D, forming the consensus motif for recognition by the Grb2 SH2 domain (Songyang et al. 1994).

The substrates for LMW-PTP have not been fully studied. However, in a megakaryocyte cell line, Mancini et al. (2007) identified several substrates for the LMW-PTP, including the Fc-gamma 2A receptor. The normally cytosolic LMW-PTP translocated to membrane-rich fractions in response to FcR clustering or platelet activation (Mancini et al. 2007) and antagonised the phosphorylation of the receptor. Although not directly shown, the evidence suggests that the translocation of the phosphatase is through the Fc-associated Grb2 adaptor (see Section 1.4.4.1). These findings, combined with the data in Chapter 4 suggest a promising candidate for a phosphatase that may aid in down regulation of the Fc-receptor mediated kinase-based signalling and as such warrants further study.

The work presented here does not directly explore a role for lipid phosphatases in signal abrogation. A key step in the internalisation of large particles is the conversion of PtdIns(4,5)P_2 to PtdIns(3,4,5)P_3. We and others have shown that inhibition of PI3K recruitment (Figure 4.5) or activity (Figure 3.10) significantly inhibits phagocytosis. It is therefore superficially conceivable
that a signalling pathway leading to the recruitment and activation of PtdIns(3,4,5)P₃ phosphatases could serve to down regulate phagocytosis at later stages.

Whilst several PtdIns(3,4,5)P₃ phosphatases such as the phosphatase and tensin homolog (PTEN) have been identified in immune cells, two main points argue against this as a means of down regulating receptor signalling. Firstly, PtdIns(3,4,5)P₃ remains on the forming phagosome until closure. Only after internalisation is PtdIns(3,4,5)P₃ dephosphorylated, giving way to the formation of PtdIns(3)P, an important regulator of membrane fusion and phagosome maturation. Secondly, inhibition of the PI3K and therefore of the formation of PtdIns(3,4,5)P₃, does not inhibit the initial formation of the phagocytic cup. This is in contrast to the protein phosphatases described above, which are upstream of actin polymerisation. Based upon these points it is unlikely that PtdIns(3,4,5)P₃ phosphatases play a role in actin clearance at the phagocytic cup.

5.2.3.2 Regulation of small GTPase Localisation

Rho-family GTPases are considered the classic ‘molecular switch’ (see Figure 1.3), alternating between their active and inactive state when bound to GTP and GDP, respectively (Etienne-Manneville & Hall, 2002). Proteins that can alter the GDP-exchange activity (GEFs) or intrinsic GTPase activity (GAPs) regulate the overall activity of GTPases (see Section 1.4.5 for more details). Another important mechanism of regulation of the GTPases is localisation to the plasma membrane. Interaction with lipid membranes is mediated through the N-terminal lipid modification. This alone is insufficient to anchor GTPases to the plasma membrane and so a polybasic sequence strengthens this interaction through association with anionic lipids in the membrane (Yeung et al. 2006).

In unstimulated cells, the main sources of charge on the inner leaflet of the plasma membrane are PtdIns(4,5)P₂ and phosphatidylserine (PS). These charged lipids can produce an overall negative surface charge on the inner leaflet that allows for electrostatic interaction with polybasic domain-containing proteins (McLaughlin & Aderem, 1995).

A lack of good probes had, until recently, hampered efforts to study the localisation of PS during phagocytosis. Recently, using a fluorescent biosensor, PS that is normally present on the plasma membrane, was shown to be maintained at the phagocytic cup and on internalised phagosomes (Yeung et al. 2009). This argues against PS metabolism accounting for changes in surface charge.
during phagocytosis. We and others have shown that PtdIns(4,5)P$_2$ is hydrolysed during phagocytosis at the phagocytic cup (see Figure 3.10 and Botelho et al. 2000). Furthermore the removal of PtdIns(4,5)P$_2$ significantly lowers the surface charge at the phagocytic cup (Yeung et al. 2006). Importantly, this abrogation of surface charge will serve to redistribute proteins such as Rac that rely upon their polybasic domains for localisation.

To confirm this idea, fluorescently labelled Rac GTPases were expressed in RAW macrophages. These cells were then challenged with IgG opsonised particles as in Chapters 3 & 4. WT Rac was localised to the cup at early stages of phagocytosis in line with its role in promoting actin polymerisation and cup formation (Figure 5.3A). Interestingly, when a constitutively GTP-bound Rac was expressed (CA Rac), clearance from the phagocytic cup still occurred (Figure 5.3B). To further test the dependence upon the surface charge, the weakly anchoring myristoylation sequence was replaced with a strongly anchored doubly-acylated sequence. This anchor permanently affixes the probe to the plasma membrane and is insensitive to changes in surface charge. The membrane anchored CA Rac, still fails to clear from the forming phagocytic cup (Figure 5.3C). These findings suggest that the intrinsic GTPase activity (or indeed activation state) is not the primary driving force behind the redistribution of Rac. More likely is that surface charge is (one of) the immediate regulator(s) of GTPase localisation. Nevertheless, a role for Rac GAPs must also be contemplated.

Although this suggests a means of regulation for small GTPases, any proteins that rely upon polybasic domains or lipid-interacting domains for recruitment to the phagocytic cup can be regulated in a similar fashion. Further work to both identify surface charge-interacting regions in proteins and modulators of surface charge itself, will aid in our understanding of the regulation of these signalling pathways during phagocytosis.

5.2.3.3 Post-translational Modification of Actin

Regulation of actin is typically considered at the level of the monomer and filament. This is usually in the context of actin binding proteins that alter the structural or enzymatic nature of actin. One further level of regulation that has not been considered to this point is the metabolic regulation of actin through post-translational modification. Modulation of actin in this way could
Figure 5.3: Localisation of fluorescently labelled Rac during phagocytosis

The cDNA encoding (A) WT-Rac (B) constitutively active (Q61L) Rac or (C) constitutively active (Q61L) Rac with an H-Ras tail sequence was expressed in a RAW murine macrophage cell line. Cells were challenged with human IgG opsonised 8.31\(\mu\)m polystyrene particles (marked with asterisks, location confirmed with DIC imaging). For details see Chapter 4 Materials and Methods.
Figure 5.3

A. WT Rac

B. CA Rac

C. CA Rac (HRas tail)
conceivably regulate the clearance of actin during phagocytosis. Although there is no direct
evidence to support this idea, the possibility will be discussed below.

**Regulation by phosphorylation:** Phosphorylation is an important means of regulation in all
cells. Among other roles, the addition of a phosphate moiety can physically block interactions,
encourage autoinhibition, shield or provide charge or promote protein-protein interaction
(Pawson & Scott, 2005). In *Dictyostelium*, actin can be phosphorylated on tyrosine 53 (Baek et
al. 2008). Modification at this site interferes with, but does not completely inhibit polymerisation
into filaments. A lower propensity of the phosphorylated species to associate with filaments
effectively decreases the average on-rate for filament formation and thus favours filament
depolymerisation.

Even though this process has not been demonstrated in mammalian cells, the homology between
*Dictyostelium* and mammalian actin is highly suggestive of a similar function existing in
mammals. In slime molds, the role of this modification is thought to be that of cell shape changes
during starvation conditions. It is conceivable that in mammals, an as yet unidentified kinase
could phosphorylate actin during phagocytosis, adding to its clearance as observed in Chapter 4.
If this were the case however, then treatment with pervanadate would exacerbate this effect
leading to further clearance. This is not what is seen however, rendering this possibility remote.

**Regulation by arginylation:** Actin can also be modified through arginylation. This
modification only occurs on beta-actin and even within this group only about 40% of the actin
bears this modification (Kashina, 2006). Addition of arginine to the N-terminus of an actin
monomer is predicted to shield the weakly negative charges that are exposed at the globular
surface with bulky positive charges. The charge on an actin filament is predicted to be far away
from the monomer interaction sites and so it is unclear what effect this modification would have
on the stability of a single filament. It is likely however, that arginylation affects the packing of
multiple filaments, leading to a looser bundling of the actin network. Several actin-binding
proteins such as tropomyosin also interact with the N-terminus and so modification of this region
could serve to regulate the interactions with other proteins.

A role for arginylation in the regulation of actin during phagocytosis is possible, but as yet
unproven. It is conceivable that a subset of actin is recruited early and another later, such that
changes in regulation of a subset could lead to depolymerisation of actin at the base of the cup and not at the pseudopods. Further analysis with isoform and modification-specific antibodies will aid in further understanding this form of regulation.

**Regulation by cleavage;** Myristoylation is a form of modification whereby the 14-carbon myristic acid is conjugated to an N-terminal glycine residue. This typically occurs cotranslationally and serves to target proteins to lipid membranes in a manner dependent on a secondary targeting motif (Magee, 1990). Under conditions where endo-proteases are activated, cryptic myristoylation sites can be uncovered by the cleavage of proteins. This was first identified in the apoptotic protein BID, which becomes cleaved by caspase 8 and is subsequently myristoylated (Zha *et al.* 2000). This targets the protein to the mitochondria where it can disrupt the membrane and release cytochrome C.

This form of post-translational modification was also shown to occur for actin. When caspase activity is induced, actin cleavage produces a 15 kDa fragment with a consensus myristoylation sequence. The enzyme N-myristoyltransferase (NMT) can then add myristic acid to the fragment and target it to the mitochondria. The purpose of this targeting is unclear, given that targeting of truncated actin to the mitochondria produced no morphological changes in the cells nor did it induce apoptosis (Utsumi *et al.* 2003).

Regardless of its purpose, cleavage of actin could serve as a mechanism for regulation of actin. Even though phagocytosis is not thought to be accompanied by pro-apoptotic signalling, it is conceivable that other non-apoptotic proteases could be activated. This could serve as a mechanism to physically down-regulate actin at the phagocytic cup.

### 5.3 Measuring the Kinetics of Actin Polymerisation

#### 5.3.1 Introduction

Work from all three chapters of this thesis deals with the regulation and modulation of actin. Several different methods have been utilised including the expression of fluorescently-labelled actin monomers or the introduction of labelled actin-binding compounds such as phalloidin.
These methods are useful, however they provide only qualitative, bulk measurements of actin localisation. Kinetic measurements are desirable when studying highly regulated and dynamic events such as those seen during phagocytosis. To date however, there is no published work studying the rate of filament treadmilling or the flow rate of the actin network during phagocytosis. These sorts of measurements would provide valuable information about the rates of polymerisation and depolymerisation that regulate network growth. Furthermore this work would help in understanding the roles of factors that alter these rates such as capping and severing proteins.

5.3.2 Techniques Used to Acquire Kinetic Data

Elsewhere in this thesis, kinetic data have been acquired using microscopic techniques such as Fluorescence Recovery After Photobleaching (FRAP). By selectively and irreversibly photobleaching a fraction of fluorophores the kinetics of the remaining labelled, but unbleached, population can be studied as the two populations homogenise (for more details see Chapter 3). The limits of spatial resolution are such that, were this technique to be applied to fluorescently-labelled actin during phagocytosis, the resulting data would be averaged values of all of the filaments within the bleach region. Furthermore, it is unlikely that bulk network flow could be discerned from changes to rates of treadmilling.

An alternative to FRAP, is to utilise single particle tracking (SPT). By reducing the fluorescent labelling such that there is one (or on average, fewer) fluorophores per diffraction limited region, the dynamics of filaments can be measured. This is possible through the correlation of multiple frames in time whereby two single fluorophores moving with a similar velocity can be assumed to reside upon the same filament or at least within a contiguous network of filaments.

SPT has been applied to many systems including studying the mobility of plasma membrane receptors, lipid anchored proteins as well as soluble signalling proteins (Alcor et al. 2009, Suzuki et al. 2007). The suitability of this method for measuring polymer networks however, can be drawn into question for a number of reasons. Firstly, reducing the density of labelling inherently reduces the available signal in relation to the background noise in the system. Secondly, the result of this reduced signal is that longer exposure times or higher illumination intensities are required. This exposes the sample to higher likelyhood of phototoxicity and photobleaching as well as increasing background signal. A third side effect of sparse labelling is the reduced
information density. With only a small fraction of the subunits labelled, fewer features of interest will be present per field. Finally, SPT allows for analysis of flow information by spatially correlating features, however given the stochastic nature of photobleaching, filament kinetics (that is polymerisation and depolymerisation) cannot be readily established.

5.3.3  Fluorescent Speckle Microscopy

An elegant solution to many of these problems was introduced several years ago. If the density of labelling is increased to between 0.1-5% (much greater than that required for SPT) a random incorporation of fluorescent monomers leads to a ‘speckled’ appearance in the filament network (Waterman-Storer et al. 1998). This is caused by uneven labelling of single filaments and also by overlap of multiple nearby filaments within one diffraction limited volume. By identifying ‘speckles’ within the network, many of the problems mentioned above are abrogated. With higher labelling density, acquisition settings can be made less intense, consequently reducing photodamage. This has the added benefit of improving the signal to noise ratio. The most important improvement however, is that by studying the integrated intensity of a speckle over time, the treadmilling kinetics can be elucidated. Speckles that increase in intensity over the acquisition time can do so due to an incorporation of a fluorescent label, suggesting active polymerisation of the network in that region. Likewise, decrease in speckle intensity can be due to disassociation of fluorescent monomers from the filaments (for an excellent discussion of the technique see Danuser & Waterman-Storer, 2006). Using these method, zones of active turnover as well as network flow can and have been identified, providing both spatial and temporal kinetic information.

5.3.4  Application of FSM to Models of Phagocytosis

FSM has been used to study both actin and microtubule networks in several systems. To date, much of the work has focused upon migratory or very large flat cells (Ponti et al. 2004, Medeiros et al. 2006). This is likely due to the presence of an extremely flat lamella encompassing the underlying cytoskeletal network that is almost two-dimensional in nature. Using these models removes a lot of the problems of a three-dimensional network, where speckles can drift in and out of focus.
Perhaps surprisingly, efforts to quantify the dynamics underlying the massive and complex rearrangement of actin during phagocytosis are so far unpublished. It would therefore be beneficial to study phagocytosis using FSM in order to better understand the underlying actin rearrangements in a physiologically relevant model. Several different models are amenable for this study; the following proposals suggest potential lines of investigation in order to study the kinetics of actin during phagocytosis.

5.3.4.1 Proposal #1

**Methodology:** In order to study large scale actin rearrangements, frustrated phagocytosis will be used to look at the Fc\(\gamma\)R-mediated ‘spreading’ of RAW macrophages. This involves sedimenting suspended RAW cells onto IgG opsonised coverslips (Marshall et al. 2001). The immobilised opsonin binds to receptors on the cell surface and the cell tries to internalise the entire coverslip. This system has distinct optical advantages as the membrane is engaged at a predictable, fixed point above the coverglass. Typical FSM experiments vary in duration; however an experiment over the course of a single spreading event (lasting several minutes) should yield ample data for analysis if acquired at a suitably high frame rate.

The choice of actin probe is an important one. As the cells are being resuspended and ‘parachuted’ onto the opsonised coverslips, microinjection (whilst being much easier to get an optimal fluorophore concentration) would not provide enough labeled cells. Expressing the DNA for fluorescently labeled actin in macrophages seems the more suitable technique. One pitfall with this method would be the potential over expression of the probe (optimal levels are between 0.1 and 5% of labelled actin: Waterman-Storer & Salmon, 1999). This problem could be overcome in a number of ways. Firstly, by transiently expressing the DNA and looking at either an early or late time point of expression, when considerably lower levels of protein can be achieved. A second option is to subclone the construct into a crippled eukaryote expression vector to achieve lower basal expression. This technique has been successfully used in the past (Watanabe & Mitchison, 2002).

This system also lends itself perfectly to Total Internal Reflection Fluorescence Microscopy (TIR-FM). This optical technique relies on the photo-optical phenomenon of total internal reflection, which produces an evanescent excitatory wave which penetrates only short distances above the coverslip. The outcome of this is specific excitation of fluorophores within 200nm of
the coverslip, precisely where Fcy-induced actin rearrangements will be evident. A result of this small imaging depth is that much of the background signal seen using epifluorescent microscopy is removed (as the fluorophores simply are not excited).

**Predicted Outcome:** Many parallels have previously been drawn between the regulation of cell migration and phagocytosis (Cougoule *et al.* 2006) Indeed the molecular basis of GTPase activation and actin regulation share many facets (Fenteany & Glogauer, 2004). By this rationale, when spreading cells are analysed using FSM, it would be expected that a small zone of short lived fast moving actin would be found at the tips of extending pseudopods with stabilised areas in the established pseudopods. These two populations can be discerned by studying the kinetics of polymerisation (which will be greater at the leading edge) and the kinematics associated with network movement.

The main problem with this technique is the concern that the cell is in fact spreading (effectively migrating) instead of undergoing phagocytosis. This downfall is highlighted by a recent publication detailing a role for ‘target geometry’ in the phagocytosis of alveolar macrophages (Champion & Mitragotri, 2006). Briefly particles with a ‘critical angle’ (which is determined by the shape of the particle in contact with the phagocyte) below a certain threshold were able to be internalised, whereas particles whose contact surface to the macrophage was above this value would simply act as a surface for receptor mediated ‘zippering’ (Griffin *et al.* 1975) along that surface and not be internalised. This would be a very important first experiment to undertake. It is a technically straightforward model system and will further add to our understanding of the parallels between actin based motility and phagocytosis.

### 5.3.4.2 Proposal #2

**Methodology:** RAW cells will be stably transfected with DNA encoding monomeric cherry red actin. This probe is an optimal candidate for fluorescent imaging, having a high extinction coefficient (~72,000 M⁻¹ cm⁻¹), long bleach half-time, favourable environmental sensitivity, fast maturation time and ready incorporation into the host cell's actin cytoskeleton (Shaner *et al.* 2004). Cells will be imaged on a spinning disk confocal microscope equipped with suitable laser lines to excite optimally at 587 nm and a fast piezo motorised stage for serial z-sectioning. Cells will be imaged in 3 spatial dimensions over the time course of engagement and internalisation of 8 μm IgG-opsonised polystyrene beads. The use of large beads makes the spatial measurement of
actin much more amenable whilst still maintaining physiological relevance as macrophages are often required to internalise senescent apoptotic bodies of this size (Fadok et al. 1992). Analysis of the four dimensional movies will require a novel implementation of speckle identification (to take into account the point spread function in the third dimension), particle tracking or correlative analysis.

**Predicted Outcome:** Actin nucleation is triggered by GTPases at the base of the cup and this nucleation provide a seed for actin polymerisation at the barbed end. It is also known that actin clears at the base of the forming cup, even before phagosome closure is complete (See Chapter 4 and Swanson & Hoppe, 2004). Because previous studies have only been able to localise actin, it would be hugely significant to determine the dynamics of actin during this process. One conjecture is that a polymerised actin bundle of fixed length simply moves towards the point of closure. Alternative to this is the continual polymerisation at the leading tip of the pseudopod, with concomitant depolymerisation at the base. Elucidating this mechanism is critical to the understanding of the molecular mechanisms of pseudopod extension and thus the formation and subsequent closure of the phagosome.

5.3.4.3 Secondary Lines of Investigation

Both of the previous suggestions aim to elucidate the basal dynamics involved in Fc-mediated actin rearrangement. However, in order to further probe the mechanism by which the actin is dynamically remodeled, several further experiments can be proposed:

**Role of Myosin in Fc-mediated actin rearrangements:** The myosin family of proteins has been implicated in two main functions during phagocytosis. Firstly in the contractile activity involved in closure of the phagocytic cup (Swanson et al. 1999), secondly and more recently Myosin X was implicated in pseudopod extension (Cox et al. 2002). To this end it would be interesting to probe the effect of myosin on the elongation (i.e. actin kinetics) and turnover (i.e. actin kinematics), data that can be acquired using FSM. In order to study where myosin family proteins are exerting effects, a pharmacological inhibitor of myosin, blebbestatin (Kovacs et al. 2004) can be added to the cells before exposing them to opsonised particles or prior to sedimentation on to coated coverslips. Blebbistatin does not compete with the nucleotide binding, nor does it interfere with the interaction of myosin with actin, but slows the release of
the phosphate, pushing equilibrium to the inactive, actin-dissociated species preventing myosin from exerting a force (ibid.).

Another option is the expression of a truncated myosin or introduction of anti-myosin antibodies. These techniques were recently used in a study of myosin X whereby phagocytosis was inhibited by a failure to recruit a force generating motor protein at the site of pseudopod extension (Cox et al. 2002). Were this to be repeated, an obvious decrease in actin kinematics should be seen, potentially without any change in polymerisation rate.

**Further roles for PI 3-kinases in FcγR-mediated actin rearrangements:** The successful completion of phagocytosis is known to be reliant upon three main classes of cytoplasmic (that is non-receptor based) kinases (Cooney et al. 2001). These kinase families are dealt with in Chapter 1. The phosphatidylinositol-3-kinases (PI3Ks) play a pivotal role in the lipid metabolism and subsequent extension of the pseudopods at the phagocytic cup through a number of mechanisms (Chapter 4 and Cox et al. 1999). Interestingly pharmacological inhibition of PI3Ks inhibits phagocytosis in a particle size dependent manner (Cox et al. 1999). Indeed, when treated with wortmannin (a potent inhibitor of PI3Ks) macrophages will successfully engage large beads and form incipient cups but rarely internalise particles (Marshall et al. 2001).

Experimentally then, actin-rich pseudopods extend to surround large particles in the presence of PI3K inhibitors such as wortmannin or LY294002. At his point it is unknown what happens to the actin as a result. When pseudopod extension halts, does actin keep polymerising at the barbed end and depolymerising at the other end to maintain a steady-state equilibrium? Alternatively does all kinetic activity halt? These questions could be answered with FSM and furthermore, shed light on the mechanisms by which actin can be regulated during phagocytosis.

### 5.4 Modulation of Host Cell Signalling by *Salmonella*

#### 5.4.1 Phospholipid Modulation During Invasion and Potential Roles for PtdIns(5)P

In Chapter 2 we utilised the lipid phosphatase SigD, from *Salmonella* to study the regulatory roles of PtdIns(4,5)P₂ in epithelial cells. As well as characterising a new tool for the modulation
of phospholipids in cells, we learned about potential roles for SigD in *Salmonella*-induced diarrhoea and virulence. Part of the characterisation of SigD function was the elucidation of its *in vivo* substrates and products. Elucidating this activity is important because it is the enzymatic activity of SigD that is responsible for its virulence (Norris *et al*. 1998).

To understand how a lipid-modifying capacity is important to the survival of *Salmonella*, it is first important to understand the role of phospholipids during *Salmonella* invasion. After internalisation, the bacteria survive and replicate inside the *Salmonella*-Containing Vacuole (SCV). This replicative niche acquires PtdIns(3)P, a typical marker of early endosomal compartments. Despite the accumulation of this lipid the SCV fails to fuse with lysosomes, maintaining a replicative niche for *Salmonella*.

When Hernandez *et al*. (2004) infected cells with *Salmonella* lacking SigD or rescued with a catalytically inactive SigD, PtdIns(3)P was found only to briefly associate with the SCV. This was unexpected given the previously published 4-phosphatase activity of SigD. In an attempt to characterise the activity of SigD the same group quantified the lipid composition of cells in each of the infection conditions. Studying the profiles of singly and doubly phosphorylated phosphatidylinositols, they suggested that SigD was able to dephosphorylate both PtdIns(3,4)P$_2$ and PtdIns(3,4,5)P$_3$ to form the PtdIns(3)P that was seen on the SCV.

Whilst this assessment is in good agreement with the reported substrate specificity *in vitro* (Marcus *et al*. 2001), there are several inconsistencies with this conclusion. Firstly, several groups have shown a SigD-dependent activation of Akt during *Salmonella* infection (Steele-Mortimer *et al*. 2000, Marcus *et al*. 2001). Recruitment of Akt to the membrane, where it can be activated is mediated through PH domains associating with PtdIns(3,4)P$_2$ and PtdIns(3,4,5)P$_3$ at the plasma membrane (see Table 3). If these lipids are being actively down-regulated by SigD, it is unclear how Akt can still become activated in a SigD-dependent fashion. Secondly, more recent work has reported a depletion of PtdIns(4,5)P$_2$ during invasion of cells by *Salmonella*. Importantly, this is not seen in the absence of SigD (Terebiznik *et al*. 2002). In Chapter 2 we further support these data by showing both a depletion of PtdIns(4,5)P$_2$ and an increase in PI(5)P when SigD is expressed in epithelial cells (Figure 2.2 & Figure 2.9 respectively).
These findings pose two important questions in relation to *Salmonella* invasion and survival. Firstly how does the composition of phospholipids change during invasion. Secondly, what is the significance of PtdIns(5)P production. These two topics will be discussed in further detail below.

### 5.4.1.1 Phosphatidylinositol Changes during *Salmonella* Invasion

The earlier findings of Hernandez *et al.* (2004) utilised electrospray ionisation mass spectrometry (ESI-MS) to characterise the lipids produced and depleted during *Salmonella* invasion. As ESI-MS is based upon charge differential, it is unable to separate PtdIns(3)P from PtdIns(4)P and PtdIns(5)P. The same is true for doubly-phosphorylated phosphatidylinositols. To more precisely study phospholipid alteration during *Salmonella* invasion, Mallo *et al.* (2008) turned to a combination of phospholipid-binding probes (see Section 5.1) and HPLC.

During *Salmonella* invasion, Mallo *et al.* (2008) found an increase in monophosphorylated phosphatidylinositol, as suggested by Hernandez *et al.* (2004). Using the more precise HPLC however, they found that the increase seen was more likely the result of increased PtdIns(4)P or PtdIns(5)P, hereafter called PtdIns(4/5)P and not increased PtdIns(3)P. Using PtdIns(3)P-specific binding domains it was confirmed that this lipid is present on SCVs; however the total amount by HPLC showed only a modest increase over uninfected controls. Furthermore the decreased doubly-phosphorylated lipid was shown not to be PtdIns(3,4)P$_2$ as predicted, but PtdIns(4,5)P$_2$. As further evidence for this pathway of metabolism, the magnitude of decreased PI(4,5)P$_2$ closely matched the increase in PI(5)P, a result highly suggestive of a substrate/product relationship. Finally, a small increase in cellular PI(3,4)P$_2$ and PI(3,4,5)P$_3$ were seen only in the presence of SigD, supporting the previously described role in Akt activation.

Dephosphorylation of PtdIns(3,4)P$_2$ and PtdIns(3,4,5)P$_3$ therefore, cannot explain the production of PtdIns(3)P at the SCV. Instead, previous studies in endosomal maturation would suggest that the action of the class III PI3K could explain the formation of lipids seen at the SCV (Vieira *et al.* 2001). Interestingly, inhibition of the type III PI3K using LY294002 or wortmannin, prevented the production of PtdIns(3)P at the SCV. This work was further supported by RNA silencing of the sole type III PI3K, VPS34, which showed a similar lack of PtdIns(3)P at the SCV (Mallo *et al.* 2008).
Overall, the evidence suggests that PtdIns(3)P formation at the SCV can be explained through recruitment and activation of VPS34. The continued production of PtdIns(3,4)P₂ and/or PtdIns(3,4,5)P₃ during invasion, despite inhibition of type I and III PI3K, remains mysterious. Several explanations have been suggested and these will be discussed further in the next section.

5.4.1.2 Possible mechanisms of PtdIns(3,4,5)P₃ Production during *Salmonella* invasion

The work of Mallo *et al.* (2008) clearly shows a production of PtdIns(3,4,5)P₃ accompanying *Salmonella* invasion of cells. Several things are known about this production: Firstly it is dependent upon the presence and activity of SigD; removing this effector prevents PtdIns(3,4,5)P₃ accumulation at ruffle sites. Secondly, the PtdIns(3,4,5)P₃ that is produced requires a pool of PtdIns(4,5)P₂. If the PtdIns(4,5)P₂ at the plasma membrane is removed before invasion, no PtdIns(3,4,5)P₃ is produced. Thirdly, this production of PtdIns(3,4,5)P₃ is insensitive to inhibition of type I or III PI3Ks.

These interesting findings suggest several possibilities. As it is most likely that the PtdIns(3,4,5)P₃ is produced directly from PtdIns(4,5)P₂, a wortmannin/LY294002 insensitive PI3K could be involved. The type II PI3K isoforms are the least sensitive to inhibition by these drugs but are also the least well understood. The three identified isoforms of the type II PI3K are constitutively associated with membranes through lipid-binding PX and C2 domains (Fry, 2001). Here they act downstream of several receptors to produce 3-phosphorylated lipid products. Even though *in vitro* work only identified PI and PtdIns(4)P as substrates (Arcaro *et al.* 2000), a role for class II PI3K in the direct or indirect production of PtdIns(3,4,5)P₃ cannot be excluded.

The requirement for catalytically active SigD in the production of PtdIns(3,4,5)P₃ is highly suggestive of a role for its lipid product, PtdIns(5)P. Little is known about this lipid, given the lack of specific probes and very low levels in resting cells. Interestingly though, the production of PtdIns(5)P has been shown to activate type I PI3K in a *Shigella* infection model (Pendaries *et al.* 2006). The studies with PI3K inhibitors however, suggest that this is not the case in *Salmonella* invasion. It is conceivable that PtdIns(5)P produced at the plasma membrane could stimulate the activity of an as yet unidentified type II PI3K. This would satisfy the requirements of a PI3K-inhibitor insensitive production of PtdIns(3,4,5)P₃. Furthermore PtdIns(4,5)P₂ would
be required both for the SigD-dependent production of PtdIns(5)P and the PI3K-mediated production of PtdIns(3,4,5)P3.

An alternative explanation for these findings has also been suggested. In a model of insulin signalling, Carricaburu et al. (2003) found that increased activity of a PI(5)P-4K served to antagonise PtdIns(3,4,5)P3 levels through the action of a PtdIns(3,4,5)P3 phosphatase. This was predicted to play a regulatory role in the insulin-mediated signalling and the subsequent activation of Akt. These findings can be extrapolated to suggest that the increased PtdIns(5)P seen during Salmonella invasion could inhibit the action of PtdIns(3,4,5)P3 phosphatases. Indeed, the Shigella homolog of SigD: IpgD, has been shown to produce PtdIns(5)P and as a result, inhibit the activity of the PtdIns(3,4,5)P3 phosphatase SHIP2 (Pendaries et al. 2006) adding credence to this theory.

In order to better understand the modifications occurring during Salmonella invasion, further characterisation of the proteins involved will be required. This includes characterising a potential role for a type II PI3K, as well as further elucidating the in vivo catalytic activity and specificity of SigD itself.

5.4.2 Assessment of an Isolated System

As a major virulence factor of Salmonella spp. (Tsolis et al. 1999), it is important to understand the functional roles of SigD. In Chapter 2 we used a heterologous expression system to study the effects of this bacterial effector in a physiologically suitable epithelial model. Previous work, such as that of Marcus et al. (2001), has focused efforts on in vitro functional characterisation. Whilst somewhat enlightening, these techniques rarely recapitulate systems in cells or tissues. The aim of Chapter 2 was to characterise SigD in the presence not only of cellular concentrations of lipid substrates, but of other factors such as pH and ion concentration gradients. These other factors are rarely taken into account in in vitro systems, where large molar excesses of lipids are often used. Under these circumstances, care must be taken when drawing conclusions so as not to confuse what an enzyme can do with what an enzyme does do.

Heterologous expression is not without its own problems, the majority of which have to do with protein expression level. Using microinjected cDNA and careful control of expression time, consistent and repeatable protein levels can be obtained. The problem is that this is still likely to
represent much more protein than is injected by a single or even multiple invading bacteria. These ‘amplified’ results are useful when developing a tool for the manipulation of host pathways (see Chapter 2). These data however, are difficult to extrapolate into the context of bacterial invasion, where many effectors are translocated simultaneously and play varying roles in invasion.

*Salmonella* effectors very rarely work alone. That is not to say that one effector will directly alter the function of another, but effectors often work to functionally antagonise each other as a means of regulation. To understand this better, one must consider the intracellular niche that *Salmonella* occupy. We have shown that SigD alone can cause blebbing, rounding and the eventual extrication of epithelial cells from a monolayer (see Figure 2.4). It can be argued that this is beneficial to the bacterial population, as localised disruption of barrier function provides more opportunities for infection. At the level of a single cell however, it is preferable for bacteria to maintain as stable a replicative niche as possible. This means that after inducing ruffling and invasion, it is beneficial to ‘reset’ the cell systems, keeping the host alive as long as possible until cytolysis occurs. This control can only be achieved through the tight regulation of individual secreted effectors. A limited amount is known about this regulation, but one representative system is briefly highlighted below.

A functional role of SigD during the early stages of bacterial invasion is to induce ruffling at the site of bacterial contact with host cells (Terebiznik *et al.* 2002). This ruffling is required for virulence, as it is the method by which bacteria are taken up into cells. In *Salmonella* lacking SigD, ruffling and internalisation still occur but are attenuated approximately two-fold (Wood *et al.* 1998). The residual ruffling that is seen is due to the semi-redundant functions of two other effectors, SopE and the highly-related SopE2 (Bakshi *et al.* 2000). Together these three effectors modulate the actin cytoskeleton by regulating the activity of small GTPases. SopE and SopE2 can bind directly to Rac, Cdc42 and to a lesser extent Rho, and stimulate GDP/GTP exchange directly (Hardt *et al.* 1998, Friebel *et al.* 2001). It is likely that much of the ruffling is mediated through Cdc42, as when a dominant negative mutant of this GTPase is expressed, bacterial entry is abrogated (Chen *et al.* 1996). The link between SigD and actin rearrangement is still unclear. Contention exists regarding a direct interaction with GTPases versus actin rearrangements as a result of lipid metabolism (Patel & Galan, 2006, Mallo *et al.* 2008).
Soon after the internalisation of *Salmonella*, the cell surface ruffling ceases and the actin-rich structures are dissipated. This antagonistic action is mostly the effect of SptP, another secreted effector that counteracts *Salmonella’s* GTPase-stimulating effects. SptP bears a consensus motif found in mammalian as well as bacterial Rho-family GAPs (Scheffzek et al. 1998 and Figure 1.3). Indeed cells infected with *Salmonella* lacking SptP, or rescued with an SptP gene that is mutated in this essential motif, can initiate actin ruffling (through SigD, SopE and SopE2), but this actin-rich ruffling is never abrogated (Fu & Galan, 1999).

Overall, the interplay between secreted effectors is remarkably complex and involves regulated secretion, localisation and degradation of effectors. *Salmonella* have evolved these systems to modulate central signalling pathways in mammalian cells, allowing them to invade and survive within a wide range of cellular environments. To further our understanding of the pathogenesis of bacteria such as *Salmonella*, a combination of *in vitro* techniques and cell or animal-based models will have to be used. This is imperative in developing successful treatments and therapies for what is a universal problem in modern healthcare.
5.5 References


Copyright Acknowledgements and Data Attribution

This body of work was achieved in collaboration with a number of people:

The work of Chapter 2 was conducted in collaboration with Drs. G. Mallo and M. Terebiznik (University of Toronto, Ontario) who technically assisted the project and performed the apoptotic assays, as well as editing the final manuscript. The HPLC data was produced through a collaboration with Dr. L. Rameh (Boston Biomedical Research Institute, Massachusetts). The study would also not have been possible without the donation of plasmids and bacterial strains from the laboratories of Drs. B. Payrastre (Inserm, Toulouse), B.B. Finlay (Michael Smith Laboratories, British Columbia) and J. Brummel (University of Toronto, Ontario). This work was published in the Journal of General Physiology (2007) volume 129, 267-283.

The work of Chapter 3 was conducted in collaboration with Drs. E.F. Corbett-Nelson and J. Marshall (Ryerson University, Ontario) who initiated the project, performed the quantification of cholesterol and edited the manuscript. All data presented were from experiments repeated or originally conducted by myself. Reagents were provided by Dr. Y. Collette (Institut National de la Santé et de la Recherche Médicale, Marseille). This work was published in the Journal of Cell Biology (2006) volume 174, 255-265.

The work of Chapter 4 was conducted by myself. Several of the experiments and phagocytic assays were repeated and quantified by Mr. A. Mendaglio whilst under my supervision.