FOCAL EXOCYTOSIS OF VAMP3-CONTAINING VESICLES AT SITES OF PHAGOSOME FORMATION

by

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A thesis submitted in conformity with the requirements for the degree of Master of Science
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0-612-53328-X
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Master of Science, 2000
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Abstract

Phagocytosis involves the receptor-mediated extension of plasmalemmal protrusions, called pseudopods, which fuse at their tip to engulf a particle. Actin polymerizes under the nascent phagosome and may propel the protrusion of pseudopods. Alternatively, membrane extension could result from the localized insertion of intracellular membranes into the plasmalemma next to the particle. Here we show focal accumulation of VAMP3-containing vesicles, likely derived from recycling endosomes, in the vicinity of the nascent phagosome. Using green fluorescent protein as both a fluorescent indicator and an exofacial epitope tag, we show that polarized fusion of VAMP3 vesicles precedes phagosome sealing. It is therefore likely that targeted delivery of endomembranes contributes to the elongation of pseudopods. In addition to mediating pseudopod formation, receptor-triggered focal secretion of endosomes may contribute to polarized membrane extension in processes such as lamellipodial elongation or chemotaxis.
Acknowledgments

I am truly indebted to my co-supervisors Dr. Sergio Grinstein and Dr. William Trimble for their unlimited guidance, patience, and encouragement. I would also like to sincerely acknowledge Dr. Fred Keeley and Dr. Jane McGlade for serving on my graduate committee. I am extremely grateful to the past and present members of the Grinstein and Trimble laboratories for their help, advice, support, and for fostering a fun and stimulating environment. Finally, I would like to thank Dr. Tobias Meyer, Dr. Hsiao-Ping Moore, Dr. Alan Schreiber, Dr. Thomas Sudhof, Dr. Marino Zerial, and especially Dr. Xiao-Rong Peng for providing me with various materials.

Some of the work presented in this thesis was published in the Journal of Cell Biology, volume 149(3), pages 697-706, May 1, 2000, and is reproduced here by copyright permission from The Rockefeller University Press.
# Table of contents

Abstract...................................................................................................................................... ii
Acknowledgements .................................................................................................................. iii
Table of contents ...................................................................................................................... iv
List of abbreviations ................................................................................................................ ix
List of figures ............................................................................................................................. xi

CHAPTER 1: INTRODUCTION .................................................................................. 1

Receptor-mediated endocytosis ........................................................................................ 1
Endosome maturation ........................................................................................................ 3
Membrane targeting and fusion ....................................................................................... 5
  Membrane fusion ............................................................................................................. 5
  NSF and SNAPs ............................................................................................................... 5
  SNAREs .......................................................................................................................... 7
  VAMP ............................................................................................................................. 7
  Syntaxin .......................................................................................................................... 8
  SNAP-25 ........................................................................................................................ 9
  Mechanism of membrane fusion ................................................................................... 9
  Regulation of membrane fusion .................................................................................... 13
Membrane Targeting ......................................................................................................... 13
  Rab GTPases .................................................................................................................. 14
  Rab effectors .................................................................................................................. 14

Phagocytosis ..................................................................................................................... 16
  Fc receptors .................................................................................................................... 16
    Signal transduction ...................................................................................................... 21
      Tyrosine kinases ....................................................................................................... 21
        Src .......................................................................................................................... 22
        Syk .......................................................................................................................... 23
      Phosphoinositide-3-kinase ..................................................................................... 23
      Phospholipase Cg ...................................................................................................... 25

Actin dynamics during phagocytosis .............................................................................. 26
  Rho family of proteins .................................................................................................... 26
    Rho family involvement in Fc receptor-mediated phagocytosis ............................. 29
    Rho family signal transduction ................................................................................ 30

Phagosome maturation ..................................................................................................... 32
Engineered phagocytic cells ................................................................. 34
Rationale and hypothesis ................................................................. 36

CHAPTER 2: MATERIALS AND METHODS ............................................. 40
  Materials and media ................................................................. 40
  Constructs ................................................................. 40
  Cell culture and transfection .................................................. 41
  Phagocytosis ................................................................. 42
  Fluorescence labelling and analysis ....................................... 43
  Phagosome isolation .......................................................... 43
  SDS-PAGE and immunoblotting ............................................. 44

CHAPTER 3: RESULTS ............................................................................ 45
  VAMP3 is present in phagocytes and becomes enriched in phagosomal membranes .... 45
  VAMP3-GFP localizes to the recycling endosomes ....................... 45
  Redistribution of VAMP3-GFP during phagocytosis ..................... 53
  Increased surface exposure of VAMP3 during phagocytosis .......... 53
  Changes in cell surface area during phagocytosis ....................... 59
  VAMP3 accumulation does not require calcium ......................... 60
  VAMP3 accumulation is sensitive to wortmannin and cytochalasin .... 60
  Distribution of LAMP1 during phagocytosis .............................. 66

CHAPTER 4: DISCUSSION ................................................................. 70
  Membrane dynamics during phagosome formation ..................... 70
    Trafficking components involved in membrane delivery ............ 71
    ARF6 ................................................................. 74
  Mechanisms of membrane accumulation at sites of phagosome formation .......................... 75
    Local decrease in endocytosis ........................................... 76
    Local increase in exocytosis ............................................. 77
    Role of microtubules ....................................................... 80
  Other cellular processes potentially mediated by focalized VAMP3 delivery .......... 84

REFERENCES .......................................................................................... 86
List of abbreviations

AP: adaptor protein
BoTx: botulinum toxin
CHO-IIA: Chinese hamster ovary cells stably transfected with FcγRIIA receptors
DAG: diacylglycerol
EEA1: early endosome-associated antigen-1: early endosomal antigen 1
FcεR: opsonin receptor that recognizes the Fc domain of immunoglobulin E
FcγR: opsonin receptor that recognizes the Fc domain of immunoglobulin G
GAP: GTPase-activating protein
GDI: GDP-dissociation inhibitor
GEF: guanine nucleotide exchange factor
GFP: green fluorescent protein
IP3: inositol-1,4,5-triphosphate
ITAM: immunoreceptor tyrosine activation motif
LAMP: lysosome-associated membrane protein
LDL: low density lipoprotein
M6PR: mannose-6-phosphate receptor
NSF: N-ethylmaleimide sensitive factor
PH: pleckstrin homology
PI: phosphatidylinositol
PI3K: phosphoinositide-3-kinase
PI3P: phosphatidylinositol-3-phosphate
PI4P: phosphatidylinositol-4-phosphate
PIP2: phosphatidylinositol-4: 5-bisphosphate
PIP3: phosphatidylinositol-3,4,5-trisphosphate
PI4P5K: phosphatidylinositol-4-phosphate 5-kinase
PLC: phospholipase C
PM-GFP: membrane-targeted form of GFP
RBC: red blood cell
SNAP: soluble N-ethylmaleimide sensitive factor attachment protein
SNAP-25: synaptosome-associated membrane protein of 25 kDa
SNARE: soluble N-ethylmaleimide sensitive factor attachment protein receptor
TeTx: tetanus toxin
t-SNARE-target SNARE
VAMP: vesicle-associated membrane protein
V-ATPase: vacuolar ATPase proton pump
v-SNARE: vesicular SNARE
### List of figures

1.1. Mechanism of SNARE-mediated membrane fusion .................................................. 11

1.2. Receptor-mediated endocytic pathway ....................................................................... 17

1.3. Signalling pathways activated during phagocytosis .................................................. 19

1.4. Plasma membrane reorganization during phagocytosis ........................................ 27

1.5. VAMP3-GFP fusion protein and its orientation in cell membranes .......................... 38

3.1. Detection of TeTx-sensitive VAMP proteins in macrophage cell lines .................... 46

3.2. VAMP3 is enriched in early phagosomes .................................................................. 48

3.3. Localization and recycling of VAMP3 in CHO-IIA cells ......................................... 51

3.4. Localization of VAMP3-GFP in CHO-IIA cells during phagocytosis .................... 54

3.5. Surface exposure of VAMP3-GFP in CHO-IIA cells during phagocytosis .............. 57

3.6. Effect of wortmannin on VAMP3-GFP redistribution during phagocytosis .......... 62

3.7. Effect of cytochalasin on VAMP3-GFP redistribution during phagocytosis .......... 64

3.8. Localization of LAMP1 in CHO-IIA cells during phagocytosis ............................... 68

4.1. Focal exocytosis of VAMP3-containing vesicles at sites of phagosome formation . 72

4.2. Hypothetical model of microtubule-dependent VAMP3-containing vesicle targeting
    to sites of phagosome formation .................................................................................. 82
CHAPTER 1
INTRODUCTION

Phagocytic cells such as macrophages and neutrophils are essential elements in the immune defense against invading microorganisms. They kill microorganisms in a number of ways, including the generation and release of toxic oxygen products by activated NADPH oxidases (Nauseef, 1999) or by engulfing the pathogen in a process called phagocytosis (Aderem and Underhill, 1999).

Phagocytosis is a type of endocytosis, a collective term describing the uptake of extracellular material into intracellular membrane-bound vesicles (Mukherjee et al., 1997). In the case of phagocytosis, the vesicle generated is termed a phagosome. Though the types of endocytosis differ with respect to size of material that can be internalized and the molecular mechanisms mediating the internalization, they are similar in that the generated vesicles mature and acquire enzymes that ultimately degrade the internalized material.

In this thesis I will describe studies aimed at determining the mechanisms controlling the formation of the phagosome. Before proceeding to detail these mechanisms, I will outline how an endocytic vesicle is generated through the receptor-mediated endocytic pathway, and how it matures into a degradative organelle, as this process is analogous to the maturation of a phagosome.

RECEPTOR-MEDIATED ENDOCYTOSIS

The best characterized type of endocytosis is receptor-mediated (Marsh and McMahon, 1999), where extracellular fluid, solutes, and membrane receptors with their associated ligands are internalized in a clathrin-dependent process. Clathrin, composed of three 190 kDa heavy chains and three 25 kDa light chains, has a three-legged “triskelion” structure that can
oligomerize into a polygonal network on the cytoplasmic face of the plasma membrane, deforming it into an invagination or pit (Marsh and McMahon, 1999; Ungewickell and Branton, 1981). However, these clathrin triskelions do not bind to membranes directly, but rather do so through adaptor proteins (APs) (Hirst and Robinson, 1998). AP2, which is involved in the formation of clathrin-coated pits at the plasma membrane, is a complex consisting of four protein subunits called adaptins: α and β2 are ~100 kDa, μ2 is ~50 kDa, and σ2 is ~20 kDa (Hirst and Robinson, 1998). The α and β2 adaptins of AP2 contain clathrin-binding sites and thus may mediate its association with the cytoplasmic face of the plasma membrane (Gallusser and Kirchhausen, 1993; Goodman and Keen, 1995). Plasma membrane receptors are concentrated in clathrin-coated pits (Hirst and Robinson, 1998). The cytoplasmic domains of these receptors contain signal sequences that interact with APs (Heilker et al., 1999; Kirchhausen et al., 1997). For example, some receptors contain a YXXØ sequence (where X is any amino acid and Ø is a large hydrophobic amino acid) (Bonifacino and Dell'Angelica, 1999) that interacts with the μ2 subunit of AP2 (Boll et al., 1996; Ohno et al., 1995). The transferrin receptor, whose ligand transferrin binds Fe³⁺, has such a signal sequence and thus becomes concentrated in clathrin-coated pits (Bonifacino and Dell'Angelica, 1999).

Invaginated clathrin-coated pits are pinched off the plasmalemma to form intracellular vesicles. This fission event is thought to be dependent on the GTPase dynamin (Schmid et al., 1998), since cells containing a dynamin mutant that is unable to hydrolyze GTP are defective in endocytosis at the vesicle fission stage (Schmid et al., 1998). Furthermore, dynamin is localized to the necks of invaginated clathrin-coated pits (Takei et al., 1995). Dynamin recruitment to clathrin-coated pits is dependent on the multidomain protein amphiphsyn which binds to the α-
adaptin of AP2 and has an SH3 domain that binds to a proline-rich domain in dynamin (David et al., 1996).

**ENDOSOME MATURATION**

Vesicles formed through receptor-mediated endocytosis go on to fuse with the sorting compartment of the early endosomes. The sorting endosomes are dispersed throughout the periphery of cells and can be identified by the enrichment in the peripheral membrane proteins Rab4 and Rab5 (Chavrier et al., 1990; Van Der Sluijs et al., 1991), members of the Rab family of small GTPases that mediate membrane trafficking (discussed later in detail). In the sorting compartment the internalized molecules are either recycled back to the plasma membrane or routed towards degradative compartments. This sorting is mediated by both the pH and morphology of the compartment. The pH of the compartment is slightly acidic (pH of ~6.0) and is maintained, as in all endosomal compartments, by the vacuolar ATPase (V-ATPase) which pumps H\(^+\) ions into the lumen of the compartment (Futai et al., 2000; Mellman et al., 1986). At the acidic pH found in endosomes, many pH-sensitive ligand-receptor complexes are dissociated (Davis et al., 1987). In the case of the transferrin receptor, transferrin remains bound, but the Fe\(^{3+}\) bound by the transferrin is released (Dautry-Varsat et al., 1983). The tubulovesicular morphology of the compartment is believed to mediate the sorting of the membrane proteins that are to be recycled from the ligands that are to be degraded (Dunn et al., 1989; Mayor et al., 1993). Since the majority of the membrane of the compartment is in the tubular extensions, most of the membrane proteins will accumulate in these tubules. Conversely, most of the volume of the compartment is in the vesicular region and thus the ligands become concentrated here. The tubules, containing the majority of the recycling receptors, bud from the sorting
compartment and return the receptors to the plasma membrane. These recycling endosomes, also classified as early endosomes, will be discussed further below. The remaining vesicular region, sometimes referred to as an endosome carrier vesicle, contains material that is to be degraded (Gruenberg et al., 1989). This compartment translocates along microtubules (Aniento et al., 1993; Gruenberg et al., 1989) to the perinuclear cytoplasm and it acquires the characteristics of a late endosome (Dunn and Maxfield, 1992; Stoorvogel et al., 1991).

Late endosomes are localized primarily in the perinuclear region. These compartments are enriched in a number of proteins, including Rab7 (Chavrier et al., 1990), Rab9 (Lombardi et al., 1993), highly glycosylated membrane proteins called lysosome-associated membrane proteins (LAMPs) (Akasaki et al., 1995), and the mannose-6-phosphate receptor (M6PR) (Griffiths et al., 1988), which delivers lysosomal enzymes from the Golgi. Due to the acidity of this compartment (pH of 5.0 - 6.0) and the presence of lysosomal enzymes, it is thought that degradation of material can be initiated in this compartment (Kornfeld and Mellman, 1989). Eventually, late endosomes are transformed into lysosomes, also in the perinuclear cytoplasm. Lysosomes can be identified by the presence of LAMPs, but the absence of the M6PR (Kornfeld and Mellman, 1989). Importantly, these are concentrated with degradative acid hydrolases, and at the pH of ~5.0 that prevails in lysosomes, provide an optimal environment for the function of these enzymes, resulting in the efficient degradation of internalized material (Kornfeld and Mellman, 1989).

The recycling endosomes are less acidic (pH ~6.5) than the sorting endosomes (Yamashiro et al., 1984). In many cell types, the recycling endosomes have a distinct juxtanuclear localization which is dependent on an intact microtubule network (Yamashiro et al., 1984). However, in some cell types the recycling endosomes are scattered throughout the
periphery (Cox et al., 2000). No matter where they are localized in the cell, the recycling endosomes can be easily identified by their enrichment in Rab11 (Ullrich et al., 1996). They ultimately fuse with the plasma membrane, returning the recycling receptors for subsequent rounds of ligand-binding and intracellular trafficking.

**MEMBRANE TARGETING AND FUSION**

The transport of membrane and luminal contents between intracellular membrane compartments is essential not only in the endocytic pathway for the maturation of an early endosome to a lysosome, but also the secretory pathway. Transport is achieved when vesicles budding from a donor organelle find their acceptor organelle and fuse, in order to deliver their contents. Membrane trafficking, which involves both the targeting and fusion between compartments, is accomplished in the cell using a variety of protein families, which are discussed below.

**MEMBRANE FUSION**

**NSF and SNAPs**

The N-ethylmaleimide-sensitive factor (NSF) protein is an ATPase originally identified as being essential for the transport of vesicles between cisternae of the Golgi *in vitro* (Malhotra et al., 1988). This protein was essential for vesicle fusion, as its depletion from the cytosol led to the accumulation of transport vesicles at the Golgi membrane (Orci et al., 1989). NSF was found to be homologous to Sec18p in yeast, a protein necessary for endoplasmic reticulum (ER) to Golgi transport, which suggested that the function of NSF in vesicle fusion is conserved and that it may have a role in vesicle fusion at different trafficking steps (Wilson et al., 1989).
However, NSF was unable to bind directly to purified Golgi membranes, but rather required the addition of cytosol (Weidman et al., 1989). This led to the purification of α, β, and γ soluble NSF-attachment proteins (SNAPs) (Clary et al., 1990), each of which could mediate NSF-binding to Golgi membranes. SNAP activity was also essential for vesicle fusion (Clary et al., 1990) and α-SNAP was found to be homologous to Sec17p in yeast, which suggested that its role in fusion is also conserved (Clary et al., 1990). SNAPs bound to integral membrane proteins in the Golgi (Whiteheart et al., 1992) and NSF could only bind SNAP when it was bound to these proteins (Wilson et al., 1992). In detergent extracts of Golgi membranes, NSF and SNAP assembled into multisubunit particles that sedimented at 20S (Wilson et al., 1992) and disassembled upon NSF-catalyzed hydrolysis of ATP.

Sollner et al. (Sollner et al., 1993b) believed that the 20S particles contained all the proteins necessary for vesicle fusion (Wilson et al., 1992), and set out to identify the integral membrane components using an affinity purification system (Sollner et al., 1993b). Using recombinant epitope-tagged NSF, SNAPs, and a detergent extract of mammalian brain, 20S particles were formed by stabilizing NSF in its membrane-bound, SNAP-associated form by incubating with the ATP analogue ATPγS that cannot be hydrolyzed. The particles were purified through attachment to a solid matrix, ATP was added, and the SNAP receptors (SNAREs) were eluted upon ATP hydrolysis by NSF. Three distinct SNAREs were released by this process, all of which had been previously identified as synapse-associated proteins essential for regulated exocytosis, i.e. fusion of synaptic vesicles at the presynaptic membrane. The SNAREs isolated were Vesicle-Associated Membrane Protein (VAMP) or synaptobrevin (Baumert et al., 1989; Trimble et al., 1988), which had been localized to synaptic vesicles,
syntaxin (Bennett et al., 1992) and synaptosome-associated membrane protein of 25 kDa (SNAP-25) (Oyler et al., 1989), which were localized to the presynaptic membrane.

When the above SNAREs were first isolated, Sollner et al. (Sollner et al., 1993b) found that homologues of both VAMP and syntaxin were found in yeast and were localized to vesicle and target membranes, respectively (Bennett and Scheller, 1993). These proteins were known to be involved in various constitutive trafficking steps in the secretory pathway, each of which was also previously found to be dependent on NSF (Graham and Emr, 1991). Therefore, Sollner et al. (Sollner et al., 1993b) proposed that the SNAREs isolated were members of a family that were distributed in a compartment-specific manner, and that membranes destined to fuse together would contain complementary SNAREs: Vesicular (v-) SNAREs, homologues of VAMP, would be present on transport vesicles and target (t-) SNAREs, homologues of syntaxin or SNAP-25, would be present on target membranes. Together with NSF and SNAPs, they proposed that the pairing of complementary SNAREs could have a role in both constitutive and regulated membrane fusion events within the cell, the mechanism of which will be discussed later.

**SNAREs**

**VAMP**

VAMPs were originally identified as components of synaptic vesicles (Baumert et al., 1989; Trimble et al., 1988). Members of this family are integral membrane proteins of around 120 amino acids with their transmembrane domain located near their carboxyl terminus such that the majority of the protein is cytosolic. Their importance in membrane fusion was known even before their isolation as SNAREs because VAMP2 is a target for certain Clostridium neurotoxins
(botulinum toxin, BoTx, and tetanus toxin, TeTx) (Schiavo et al., 1992). These neurotoxins are proteases which, through the recognition of specific peptide bonds, selectively cleave proteins involved in synaptic vesicle exocytosis, thus inhibiting neurotransmission (Rossetto et al., 1994).

More specifically, some VAMPs are substrates for TeTx and BoTx B, D, F, and G (Montecucco and Schiavo, 1994). These toxins have proven extremely useful in that they have also identified the importance of VAMP2 in regulated secretion from a variety of nonneuronal cell types, such as the exocytosis of zymogen granules in calcium-stimulated pancreatic acinar cells (Gaisano et al., 1994) or GLUT4-containing vesicles in insulin-stimulated adipocytes (Olson et al., 1997).

While VAMP2 is specifically expressed in secretory cells and involved in regulated secretion, other VAMPs are widely expressed and may be involved in constitutive membrane trafficking events. VAMP3, also toxin-sensitive, is ubiquitously expressed and localized to the recycling endosomes (McMahon et al., 1993). When cells are microinjected with tetanus toxin, the rate of transferrin recycling decreases, suggesting that the fusion of recycling endosomes with the plasma membrane is regulated by VAMP3 (Galli et al., 1994). Other VAMPs that have been identified are localized to various compartments in the cell, including the Golgi (Steegmaier et al., 1999) and endosomes (Wong et al., 1998b), and may be involved in constitutive membrane trafficking from these compartments.

Syntaxin

Syntaxin was originally identified as a component of the presynaptic membrane of neurons (Bennett et al., 1992; Inoue et al., 1992). Members of this family are integral membrane proteins of around 300 amino acids with their transmembrane domain located near their carboxyl terminus such that the majority of the protein, as in the case of VAMP, is cytosolic. The ability
of BoTx C to cleave syntaxin1 had already proven its involvement in membrane fusion (Blasi et al., 1993b). Whereas syntaxin1 is specifically expressed in secretory cells and involved in regulated secretion, other syntaxin family members are ubiquitously expressed and localized to the plasma membrane (Hackam et al., 1998), Golgi (Bock et al., 1997), and endosomal compartments (Prekeris et al., 1998; Wong et al., 1998a), and are likely to be involved in constitutive membrane trafficking between compartments.

SNAP-25

As with syntaxin, SNAP-25 was also originally identified as a component of the presynaptic membrane of neurons (Oyler et al., 1989). However, unlike syntaxin and VAMP, SNAP-25 is not an integral membrane protein, but instead is anchored to the membrane through palmitoylation (Veit et al., 1996). Its importance in membrane fusion was realized when it was found to be a substrate of BoTx A and E (Binz et al., 1994; Blasi et al., 1993a).

Whereas SNAP-25 is expressed mainly in neurons and participates in regulated exocytosis, synaptosome-associated membrane protein of 23 kDa (SNAP-23) is a ubiquitously expressed isoform that may participate in constitutive membrane trafficking events in other cell types (Ravichandran et al., 1996).

Mechanism of membrane fusion

The SNARE hypothesis proposed that membranes destined to fuse together would contain complementary SNAREs, with v-SNAREs present on transport vesicles and t-SNAREs on target membranes (Sollner et al., 1993b). It was found in vitro that VAMP, syntaxin and SNAP-25 could spontaneously assemble into a stable ternary complex (Sollner et al., 1993a),
and that this complex could bind SNAPs and NSF to form a 20S particle. NSF-dependent ATP hydrolysis was able to dissociate the complex (Sollner et al., 1993a), and it was proposed that it would also promote membrane fusion (Sollner et al., 1993a). This idea has since been challenged: With the use of an *in vitro* yeast vacuolar homotypic fusion assay, Nichols et al. (Nichols et al., 1997) found that complementary SNAREs were necessary on the membrane compartments for fusion, but that NSF was not.

It has now been proposed that the formation of the SNARE complex, not its disassembly, leads to membrane fusion (Chen et al., 1999; Nichols et al., 1997). Structural information of the complex derived through fluorescence resonance energy transfer experiments, crystallography, and electron microscopy (Hanson et al., 1997; Lin and Scheller, 1997; Sutton et al., 1998) have shown that the SNAREs are aligned in parallel to form a four-helix complex, with their membrane anchors extended from one end. The helicity of each individual SNARE is significantly enhanced upon association in the complex (Fasshauer et al., 1997) and the release of energy upon formation of such a stable complex may be enough to drive membrane fusion (Fasshauer et al., 1997; Hanson et al., 1997; Sutton et al., 1998). In fact, it has now been demonstrated, using an *in vitro* fusion assay between liposomes, that the SNAREs alone can lead to membrane fusion (Weber et al., 1998). As for the roles of NSF and SNAPs in the fusion process, it is believed that their binding and subsequent NSF-dependent ATP hydrolysis is necessary only for the dissociation of the SNAREs from one another, allowing them to be recycled for subsequent rounds of fusion (Nichols et al., 1997). A summarization of SNARE-mediated membrane fusion is depicted in Fig. 1.1.

There has also been a suggestion that the SNARE complex is not directly involved in the fusion process, but rather complex assembly may generate a signal that leads to membrane
Figure 1.1. Mechanism of SNARE-mediated membrane fusion. (a-b) Once a vesicle has been targeted to its acceptor membrane, the v-SNARE VAMP on the vesicle and the t-SNAREs syntaxin and SNAP-25 on the acceptor membrane interact to form a four-helix complex. (c) The energy derived from this complex formation is thought to drive membrane fusion. (d) The SNARE complex is then bound by SNAP, which is followed by NSF binding. NSF-dependent hydrolysis dissociates the complex, allowing the SNAREs to be recycled for subsequent rounds of fusion.
fusion (Peters and Mayer, 1998; Ungerma nn et al., 1998). This is supported by in vitro yeast vacuolar homotypic fusion assays, where it has been found that the SNARE complex can be disassembled before membrane fusion occurs (Ungerma nn et al., 1998).

**Regulation of membrane fusion**

In addition to SNAPs, other proteins can also bind to SNAREs and may regulate the assembly of SNARE complexes and thus membrane fusion. Members of the Sec1p family in yeast (Novick et al., 1980) and their homologues in mammalian cells, members of the nSec1/munc18 family (Hata et al., 1993; Pevsner et al., 1994b), have been implicated in both constitutive and regulated membrane trafficking steps (Halachmi and Lev, 1996). Though these proteins are soluble, they can become membrane-associated due to their ability to bind to the t-SNARE syntaxin (Hata et al., 1993; Pevsner et al., 1994a). Such an association prevents syntaxin from interacting with VAMP and SNAP-25 (Pevsner et al., 1994a). It has therefore been proposed that these proteins are negative regulators of SNARE complex assembly and thus membrane fusion (Pevsner et al., 1994a).

**Membrane Targeting**

While SNAREs are thought to mediate fusion between membrane compartments, the targeting of transport vesicles, which refers to their translocation towards, and docking at, the acceptor membrane is thought to be regulated by the Rab family of small GTPases (Pfeffer, 1999). A brief description of this family and how they may mediate targeting follows below.
**Rab GTPases**

The Rab family, part of the Ras superfamily of small GTPases, contains numerous members that are localized to specific membrane compartments (Martinez and Goud, 1998; Novick and Zerial, 1997). Rab proteins, as with all other small GTPases, cycle between an active GTP- and inactive GDP-bound form. They are regulated by guanidine nucleotide exchange factors (GEFs) (Horiuchi et al., 1997), which promote the exchange of GDP for GTP, and by GTPase-activating proteins (GAPs) (Albert and Gallwitz, 1999), which promote the hydrolysis of GTP to GDP. The association of Rabs with membranes, which is mediated by their prenylation, is dependent on their nucleotide status. Rab-GDP is found in the cytosol complexed with a GDP-dissociation inhibitor (GDI) (Pfeffer et al., 1995; Ullrich et al., 1993). When this complex is targeted to a membrane, a GDI displacement factor (GDF) dissociates GDI from Rab-GDP (Dirac-Svejstrup et al., 1997), and a GEF exchanges the GDP for GTP (Horiuchi et al., 1997). It is in this nucleotide state that Rab is able to recruit proteins, referred to as Rab effectors, to the transport vesicle to target them to the acceptor membrane.

**Rab Effectors**

Rabs may mediate the translocation of transport vesicles to their acceptor membrane through association with motor proteins that move along the actin and/or microtubule-based cytoskeleton. For instance, GTP-bound Rab6 is localized to the Golgi and can interact with a protein, named Rabkinesin6, that is similar to the motor protein kinesin (Echard et al., 1998). Rab6, through the recruitment of Rabkinesin6, may allow the translocation of vesicles on the microtubule-based cytoskeleton towards their target membrane. Other Rabs may have similar effectors.
Rabs may mediate vesicle targeting by recruiting proteins that dock vesicles at their acceptor membrane. Rab5 regulates the fusion of newly-budded clathrin-coated vesicles (CCVs) with early endosomes and is also involved in early endosome-early endosome fusion (Gorvel et al., 1991). A Rab5 effector protein is the early endosomal antigen 1 (EEA1) (Simonsen et al., 1998). EEA1 is a multidomain protein that contains two binding sites for Rab5, one on each end, and an FYVE domain that binds to the membrane phospholipid phosphatidylinositol-3-phosphate (PI3P) (Gaullier et al., 1998). It has been speculated that EEA1 binds to both Rab5 and PI3P on one early endosome, and using its other Rab5 binding site, is able interact with another Rab5-containing early endosome, docking the two together (Christoforidis et al., 1999).

Another docking complex is the multisubunit Exocyst or Sec6/8 complex that is localized to sites of exocytosis in both yeast and mammalian cells (TerBush et al., 1996). In yeast, where it is localized to sites of bud growth (TerBush et al., 1996), the Exocyst component Sec3p specifies the site of, and may initiate, complex formation on the plasma membrane (Finger et al., 1998). The yeast Rab protein Sec4p, found on vesicles emerging from the Golgi complex, can interact with the Sec15p component of the Exocyst (Guo et al., 1999), and likely docks these vesicles at the plasma membrane (Guo et al., 1999).

In addition to targeting vesicles to their destination, Rabs may also regulate the formation of SNARE complexes. For example, Rabs and their effectors may mediate the release of Sec1p family members from t-SNAREs. As previously mentioned, these proteins negatively regulate SNARE complex formation by binding to t-SNAREs, interfering with SNARE complex formation. In support of this, the yeast Rab Vps21p, involved in Golgi to vacuole transport (Horazdovsky et al., 1994), has an effector Vac1p that is able to bind to the Sec1p homologue Vps45p, which associates with the t-SNARE Vps6p involved in this pathway (Peterson et al.,
1999). Furthermore, the necessity of the yeast Rab Yptlp, involved in ER to Golgi transport, is bypassed by mutations in the Sec1p homologue Slylp that binds to the t-SNARE Sed5p involved in this trafficking step (Dascher et al., 1991). Yptlp itself has been found to interact with Sed5p and this is associated with the displacement of Slylp from Sed5p (Lupashin and Waters, 1997).

Receptor-mediated endocytosis results in the internalization of extracellular fluid, solutes, and membrane receptors and their associated ligands. The resulting endosome matures through sequential membrane targeting and fusion events which are dependent on Rabs and SNAREs, respectively. Through these processes, summarized in Fig. 1.2, the endosome is transformed into a lysosome where the internalized contents are ultimately degraded.

**PHAGOCYTOSIS**

As previously mentioned, phagocytosis is the internalization of solid particles, often microorganisms, into membrane-bound vacuoles called phagosomes. This process is receptor-mediated and initiates a variety of signals that results in the extension of membranes or pseudopods that engulf the particle. This section will describe the mechanisms involved in phagocytosis, depicted in Fig. 1.3, that lead to the generation of a phagosome.

**Fc RECEPTORS**

There are two types of receptors that are capable of initiating phagocytosis (Aderem and Underhill, 1999). One type can bind directly to the surface of the invading pathogen through the recognition of specific surface molecules such as sugars. The other type of receptors bind indirectly through host cell proteins called opsonins, which include immunoglobulins (Igs) and complement proteins, that coat the surface of invading pathogens.
Figure 1.2. Receptor-mediated endocytic pathway. Extracellular fluid, solutes, and membrane receptors with their associated ligands are internalized into clathrin-coated pits. The resulting vesicles fuse with the sorting compartment of the early endosome, where ligands dissociate from their receptors due to the acidity of the compartment. The recycling receptors are routed to the recycling compartment of the early endosomes whereas the ligands are routed via endosomal carrier vesicles to the late endosomes. Vesicles budding from the Golgi deliver lysosomal enzymes to the late endosomes, which are ultimately transformed into lysosomes where the internalized contents are degraded. ECV: endosomal carrier vesicle; PM: plasma membrane.
PM

ligand

recycling receptor

cloathpin-coated pit

recycling endosomes

sorting endosome

ECV

late endosome

Golgi

lysosome
Figure 1.3. Signalling pathways activated during phagocytosis. The clustering of Fc receptors by opsonized particles leads to the activation of Src family kinases, which phosphorylate the ITAM motifs of the receptors, creating docking sites for the SH2 domains of Syk kinase. Some downstream effectors of the activated kinases include PI3K and the Rho family of GTPases, which affect the actin dynamics of the cell.
IgG-opsonized particle

PI3K

PM

FcγR

PIP2 → PIP3

Dbl GEF

PIP → PIP2

Gelsolin

actin monomers

actin filament

Syk

Src

Rho

CDC

WASP

Arp2/3

α5β1 integrin
Igs are Y-shaped proteins consisting of two antigen-binding [F(ab)] domains and an effector domain (Fc). They coat the surface of pathogens though the F(ab) domains, leaving the Fc domain unengaged. Fc receptors (FcRs) bind to the exposed Fc domains. The best characterized Fc receptors are those that bind to IgG (FcγRs), recently reviewed by Gessner et al. (Gessner et al., 1998). There are several classes of FcγRs: FcγRI, II, and III. These classes differ in structure, affinity for IgG, and expression level in the different phagocytic cells. However, all have a homologous extracellular domain that contains the IgG-binding site and Ig-like domains, thus classifying them as members of the Ig gene superfamily. The structures differ between classes in that FcγRIIIs are monomeric, whereas FcγRI and FcγRIII are oligomeric complexes, consisting of a ligand-binding transmembrane α chain with associated cytosolic γ chains. The clustering of receptors by IgG-opsonized particles or with antibodies against the receptors themselves generates a variety of signals.

**Signal transduction**

**Tyrosine Kinases**

The clustering of Fc receptors results in the tyrosine phosphorylation of numerous proteins (Greenberg et al., 1993; Greenberg et al., 1994) through the activation of non-receptor kinases. These phosphorylated proteins accumulate at sites of phagosome formation as revealed by immunofluorescence using anti-phosphotyrosine antibodies (Greenberg et al., 1993). The induced phosphorylation is critical since the tyrosine kinase inhibitor genistein blocks phagocytosis (Greenberg et al., 1993). Included among the phosphorylated proteins are the receptors themselves (Duchemin et al., 1994; Huang et al., 1992) (Fig. 1.3). The tyrosine residues in the receptors that are phosphorylated are arranged in the sequence
YXXL(X)\textsubscript{m}YXXL (X is any amino acid), which is called the immunoreceptor tyrosine-based activation (ITAM) motif (Samelson and Klausner, 1992). This motif is found within the cytoplasmic domain of FcγRII and in the associated cytosolic γ chains of FcγRI and FcγRIII (Indik et al., 1995). The ability of these receptors to initiate phagocytosis is dependent on the ITAM motif (Indik et al., 1995), since COS cells transfected with FcγRII are able to internalize IgG-opsonized particles, whereas transfection of a receptor that is mutated at both tyrosine residues in the ITAM inhibits phagocytosis (Mitchell et al., 1994).

\textit{Src}

Non-receptor tyrosine kinases of the Src family are thought to be responsible for the phosphorylation of the ITAMs upon receptor clustering (Fig. 1.3). Members of this kinase family have been found to be activated and associated with FcγRs in phagocytic cells upon clustering (Ghazizadeh et al., 1994; Hamada et al., 1993). Furthermore, many are capable of phosphorylating FcγRs \textit{in vitro} (Huang et al., 1992; Hunter et al., 1993). However, the most convincing evidence for their involvement in ITAM phosphorylation came with the finding that FcγRs of macrophages from mice devoid of Fgr, Hck, and Lyn (which are the most predominant members of the Src kinase family in these cells) do not get phosphorylated upon clustering (Fitzer-Attas et al., 2000). Surprisingly, phagocytosis by these macrophages is not inhibited, although the process is considerably delayed, which implies that Src family kinases are not critical for phagocytosis to occur (Fitzer-Attas et al., 2000). In addition to phosphorylating the receptors, activated Src kinases phosphorylate tyrosines on numerous other proteins (Fitzer-Attas et al., 2000). Some of the downstream effectors of Src kinases are discussed below.
The phosphorylated tyrosine residues in the ITAMs may serve as anchoring sites for the Src homology 2 (SH2) domains of the non-receptor tyrosine kinase known as Syk (Fig. 1.3), which can be immunoprecipitated with FcγRs in phagocytes following clustering (Ghazizadeh et al., 1995; Kiener et al., 1993). Syk kinase is activated upon FcγR-clustering and this is associated with phosphorylation of its tyrosine residues (Agarwal et al., 1993; Darby et al., 1994).

There is a significant amount of evidence demonstrating that Syk is essential for phagocytosis. The relatively selective Syk inhibitor piceatannol prevents FcγR-mediated phagocytosis in neutrophils (Raeder et al., 1999). Moreover, monocytes lacking Syk through the use of antisense oligonucleotides (Matsuda et al., 1996), and macrophages derived from Syk knock-out mice (Crowley et al., 1997), are similarly incapable of internalizing IgG-opsonized particles. Interestingly, chimeras of the extracellular ligand binding domain of FcγIII with an intracellular Syk kinase domain can promote phagocytosis, implying that Syk alone is sufficient to generate all the signals necessary for phagocytosis to occur (Greenberg et al., 1996). Among the downstream effectors of activated Syk may be a lipid kinase, which is reviewed next.

**Phosphoinositide-3-Kinase**

Phosphatidylinositol (PI) is an important component of the cellular phospholipids. It can be phosphorylated at several hydroxyl positions in the inositol ring (3', 4', or 5'), each by a distinct family of lipid kinases (Hinchliffe et al., 1998), to give rise to a variety of phosphorylated derivatives called phosphoinositides. Some of these include PI-3-phosphate (PI3P), PI-4-phosphate (PI4P), PI-4,5-bisphosphate (PIP2), and PI-3,4,5-trisphosphate (PIP3).
FYVE domains and pleckstrin homology (PH) domains in proteins are able to bind to specific phosphoinositides. FYVE domains bind to PI3P (Stenmark and Aasland, 1999) and PH domains can bind to the other phosphoinositides with varying specificity (Bottomley et al., 1998). The generation of specific phosphoinositides in membrane compartments by lipid kinases can affect the localization and/or activity of proteins containing these binding domains. Thus, phosphoinositides are important lipid signalling molecules, and have been found to regulate membrane trafficking, cytoskeletal architecture, and mitogenic signalling (Martin, 1998).

Several classes of kinases exist that can phosphorylate the 3' position of phosphoinositides. These kinases are collectively termed phosphoinositide-3-kinases (PI3Ks), recently reviewed by Vanhaesebroeck et al. (Vanhaesebroeck and Waterfield, 1999). Class IA PI3Ks consist of an 85 kDa regulatory subunit p85 and a 110 kDa catalytic subunit p110. They can phosphorylate the 3' position of the inositol ring, including PI and PIP2, to generate PI3P or PIP3, respectively. Class IA PI3Ks are regulated by tyrosine kinases. Activated receptor tyrosine kinases or receptor-associated tyrosine kinases can phosphorylate receptors and/or various adaptor proteins, thus generating docking sites for the SH2 domain of the p85 regulatory subunit, which results in the recruitment and activation of the p110 catalytic subunit.

The clustering of FcγRs is associated with an increase in PI3K activity that is immunoprecipitable with anti-phosphotyrosine antibodies (Ninomiya et al., 1994). PI3K may be recruited to activated FcγR receptors in a number of ways. It has been proposed that PI3K may be recruited to the activated receptors through phosphorylated Syk (Chacko et al., 1996) (Fig. 1.3), which is consistent with the decrease of immunoprecipitable PI3K with anti-phosphotyrosine antibodies in macrophages from Syk knock-out mice (Crowley et al., 1997). PI3K may also be recruited through the multidomain adaptor protein Cbl (Liu and Altman, 1998).
Cbl is recruited to FcγRs through binding of its proline-rich domain to the SH3 domain of Src kinases (Liu and Altman, 1998; Tanaka et al., 1995). Cbl is tyrosine phosphorylated during FcγR activation and this is associated with an increase in PI3K activity that is immunoprecipitable with anti-Cbl antibodies (Matsuo et al., 1996). In support of this pathway of PI3K activation, in macrophages lacking Src family kinases, the PI3K activity that is immunoprecipitable with anti-Cbl antibodies is abolished (Fitzer-Attas et al., 2000).

PI3K activity is essential for phagocytosis. Phagocytic cells incubated with wortmannin, which binds to the catalytic subunit of most PI3Ks and inhibits their function, bind particles but are unable to internalize them (Araki et al., 1996; Ninomiya et al., 1994). Thus, phosphoinositide phosphorylation by PI3K seems to play a crucial role in phagocytosis and may have an effect on actin cytoskeletal rearrangements and/or membrane trafficking, both of which have been implicated in phagocytosis and will be discussed in further sections.

**Phospholipase Cγ**

Phospholipase C (PLC) cleaves PIP2 to generate diacylglycerol (DAG) and inositol 1,4,5-trisphosphate (IP3), the latter of which mediates an intracellular rise in cytosolic calcium. The PLC isoform PLCγ is activated by tyrosine kinases. During FcγR clustering in phagocytic cells, PLCγ is tyrosine phosphorylated, IP3 is generated, and intracellular calcium levels rise (Di Virgilio et al., 1988; Liao et al., 1992; Young et al., 1984). PLCγ phosphorylation is most likely mediated by activated Src kinases, as they are capable of phosphorylating PLCγ in vitro (Liao et al., 1993). However, the role of an IP3-induced intracellular calcium rise in phagocytosis is questionable since phagocytosis stimulated by FcγRs in both neutrophils and macrophages has been demonstrated to be calcium-independent (Di Virgilio et al., 1988; Jaconi et al., 1990).
**ACTIN DYNAMICS DURING PHAGOCYTOSIS**

Signal transduction through Fc receptors ultimately leads to the reorganization of the plasma membrane, such that protrusions of membrane or pseudopods extend around the particle forming a "phagocytic cup" and ultimately fuse together at their tips to form a membrane-bound vacuole termed a phagosome. This process is believed to require the sequential interaction of unligated receptors on the surface of the cell with Igs that coat the particle such that the plasma membrane "zippers" around the particle (Griffin et al., 1975) (Fig. 1.4). Filamentous actin (F-actin) and a variety of F-actin binding proteins including α-actinin, paxillin, talin, and vinculin, accumulate in the forming phagocytic cup (Allen and Aderem, 1996; Greenberg et al., 1990). This finding, in addition to the fact that phagocytosis is inhibited by cytochalasins (Zigmond and Hirsch, 1972), drugs which bind to the barbed ends of actin filaments and therefore prevent further monomer addition, prompted the suggestion that the force generated through actin polymerization may drive the extension of plasma membrane over the particle (Fig. 1.4). It is now becoming clear which proteins are involved in mediating these actin changes during phagocytosis, and these are introduced below.

**RHO FAMILY OF PROTEINS**

The Rho family of small GTPases link extracellular signals to changes in the actin cytoskeleton (Nobes and Hall, 1995; Ridley and Hall, 1992; Ridley et al., 1992). They cycle between an active GTP-bound form and an inactive GDP-bound form, the conversion between the two being catalyzed by GEFs (Whitehead et al., 1997) and GAPs (Scheffzek et al., 1998), respectively. Three well characterized members of this family include Rho, Rac, and Cdc42,
Figure 1.4. Plasma membrane reorganization during phagocytosis. The sequential interaction of unligated receptors on the cell surface with IgS that coat the particle results in the “zippering” of plasma membrane over the particle surface. Actin polymerization accompanies phagosome formation and may drive the extension of plasma membrane over the particle surface. PM: plasma membrane.
phagosome

IgG-opsonized particle

FcyR

actin filament
each of which stimulates the formation of a distinct actin-based structure. Rho induces the formation of stress fibers (Ridley and Hall, 1992), Rac induces the formation of membrane ruffles or lamellipodia (Ridley et al., 1992), and Cdc42 induces the formation of filipodia (Nobes and Hall, 1995).

**Rho family involvement in Fc receptor-mediated phagocytosis**

Rho, Rac, and Cdc42 are also found in phagocytic cells and induce the formation of distinct actin-based structures (Allen et al., 1997). An essential role for these GTPases in phagocytosis has been established by several groups by using the C3 transferase toxin from *Clostridium botulinum*, which inactivates Rho through ADP-ribosylation, and through the use of dominant negative constructs of Rac and Cdc42 that are unable to exchange GDP for GTP.

Microinjection of the C3 toxin into J774 macrophages to inactivate Rho inhibited phagocytosis (Hackam et al., 1997). The amount of F-actin in these cells was substantially decreased, suggesting that Rho inactivation leads to F-actin depolymerization (Hackam et al., 1997). Though the cells were able to bind particles, the clustering of receptors did not occur, implying that an intact actin cytoskeleton is essential for receptor clustering (Hackam et al., 1997).

Caron et al. (Caron and Hall, 1998) found that FcγR-transfected Swiss 3T3 fibroblasts or J774 macrophages expressing dominant-negative Cdc42 or Rac bound IgG-opsonized particles but were unable to internalize them. Similarly, FcγR-mediated phagocytosis in RAW macrophages (Cox et al., 1997) and IgE receptor (FceRI)-mediated phagocytosis by mast cells (Massol et al., 1998) was inhibited by microinjection of dominant-negative proteins. However, although phagocytosis was inhibited, F-actin accumulation at sites of particle binding was only
attenuated (Cox et al., 1997; Massol et al., 1998). Further characterization of these sites with electron microscopy revealed that the morphology of the actin cups formed by each mutant was distinct (Massol et al., 1998). This implied that Cdc42 and Rac, each of which mediates the formation of distinct actin structures in these cells, must cooperate during phagocytosis to form functional actin cups that internalize the attached particles (Massol et al., 1998). The Rho GTPases are therefore essential for mediating changes in the actin cytoskeleton after particle binding to allow phagocytosis to occur.

**Rho family signal transduction**

The mechanism of Rho family recruitment and/or activation after receptor clustering is not well characterized. Immediately upstream of these GTPases in a signal transduction pathway would be a GEF. Rho GEFs belong to the Dbl family. Members of this family possess a Dbl homology domain that encodes GEF activity and a PH domain which can bind to PIP3 (Whitehead et al., 1997). PI3K may therefore serve to localize these GEFs through the production of PIP3, and could therefore be upstream of the Rho GTPases. In support of this, Rac-dependent membrane ruffling in platelet derived growth factor (PDGF)-stimulated cells is dependent on PI3K (Nobes et al., 1995) which can stimulate nucleotide exchange on Rac (Hawkins et al., 1995). As previously mentioned, PI3K is activated during phagocytosis and through production of PIP3, may serve to localize Rho GEFs at sites of phagocytosis (Fig. 1.3), including the Rac-specific GEF Vav. Vav is phosphorylated by Src kinases during phagocytosis (Darby et al., 1994; Fitzer-Attas et al., 2000), and this phosphorylation is necessary for its exchange activity (Crespo et al., 1997).
Once the Rho GTPases have been localized and/or activated at sites of phagocytosis, there are several ways in which they may mediate their effects on the actin cytoskeleton. Downstream of the Rho GTPases must be proteins that can regulate actin polymerization directly. In an unstimulated cell, existing actin filaments are prevented from continuous polymerization through the binding of capping proteins to their barbed or fast growing ends, which prevents the further addition of actin monomer. The actin-binding protein gelsolin, which also contains a PH domain that binds predominantly to PIP2, is bound to the ends of actin filaments in resting cells and is thought to prevent their elongation (Sun et al., 1999). Actin filament polymerization could therefore be stimulated through the uncapping of existing actin filaments. Rac has been found to initiate the uncapping of filaments (Hartwig et al., 1995). This may be due to its ability to stimulate PI4P5K (Tolias et al., 2000), which catalyzes the production of PIP2 from PI4P, since PIP2 synthesis is critical for Rac-mediated filament uncapping (Hartwig et al., 1995). The PIP2 produced may bind to gelsolin, releasing it from actin filament ends, thus allowing actin polymerization at the exposed ends. This is consistent with the fact that fibroblasts lacking gelsolin are defective in Rac-dependent membrane ruffling (Azuma et al., 1998). Localization and/or activation of Rac at sites of phagocytosis may be mediating actin changes through a similar mechanism (Fig. 1.3).

In addition to the uncapping of existing actin filaments, actin polymerization can also be stimulated through the nucleation of new actin filaments. The Arp2/3 multiprotein complex can nucleate actin filaments (Machesky and Gould, 1999) and this activity is stimulated by binding of Wiskott Aldrich Syndrome Protein (WASP) (Snapper and Rosen, 1999), a multidomain protein that also contains a Cdc42-binding site. Activation of Cdc42 at sites of phagocytosis could be mediating actin changes through the binding of WASP, which could recruit Arp2/3
(Rohatgi et al., 1999) (Fig. 1.3). In support of such a pathway, Arp2/3 has been found to be essential for FcγR-mediated phagocytosis (May et al., 2000).

The Rho family of proteins are responsible for the generation of distinct actin-based structures in cells through their signalling to downstream effectors that directly affect actin dynamics. Such mechanisms may be initiated at sites of phagocytosis through the localization and/or activation of Rho family members, leading to actin polymerization that may be crucial for driving the plasma membrane around particles that are to be internalized (Fig. 1.4).

**PHAGOSOME MATURATION**

In order to degrade phagocytosed material, the phagosome must mature into a degradative organelle, the phagolysosome. Such a transition is not just a single fusion step with a lysosomal compartment, but rather a dynamic and sequential maturation process in which the phagosome loses characteristics of the early endosomes and acquires those of later endosomal compartments, eventually becoming a phagolysosome. Using western blot analyses of isolated phagosomal membranes (Desjardins et al., 1994; Pitt et al., 1992), early phagosomes have been found to be enriched in early endosomal components such as the transferrin receptor and Rab5 (Desjardins et al., 1994). With time, they lose these components and become enriched in late endosomal proteins such as M6PR and Rab7 (Desjardins et al., 1994; Pitt et al., 1992). Eventually, the phagosome accumulates proteins normally enriched in lysosomal compartments, including LAMP1, LAMP2, and the acidic hydrolases cathepsin D and β-glucoronidase (Desjardins et al., 1994; Pitt et al., 1992). By this point, it has also acquired V-ATPases (Pitt et al., 1992) which create an acidic environment where the lysosomal hydrolases can function optimally. The resulting phagolysosome efficiently degrades ingested material.
The remodelling of the phagosome implies that it undergoes numerous fusion and fission events. It has been found both in vivo (de Chastellier and Thilo, 1997; Desjardins et al., 1997) and in vitro (Jahraus et al., 1998; Mayorga et al., 1991) that phagosomes can fuse with early endosomes, late endosomes, and lysosomes. However, fusion with the various compartments is dependent on the maturity of the phagosome. For instance, newly formed phagosomes fuse preferentially with early endosomes (Desjardins et al., 1997), but eventually lose this ability and begin fusing more efficiently with the late endosomes and lysosomes (de Chastellier and Thilo, 1997). Phagosome fusion with these compartments is not believed to be complete, i.e. the phagosome does not fuse with the entire organelle. Rather, according to the "kiss and run" hypothesis (Desjardins et al., 1994), phagosome maturation consists of numerous transient fusion and fission reactions, each of which result in only a brief transfer of membrane and lumen contents. Such a hypothesis is consistent with the finding that endocytic markers of varying sizes, when initially loaded into the same compartment, are delivered to the phagosome at different rates (Desjardins et al., 1997; Wang and Goren, 1987).

Since phagosomal maturation involves a series of fusion events, it is not surprising that it may be regulated by proteins involved in membrane trafficking within the cell. In vitro fusion of phagosomes with early endosomes was demonstrated to be dependent on Rab5, since anti-Rab5 antibodies and depletion of Rab5 from cytosolic fractions prevented fusion (Alvarez-Domínguez et al., 1996). NSF and SNAPs have been localized to phagosomes (Alvarez-Domínguez et al., 1997), with NSF proven to be essential for in vitro phagosome-endosome fusion, since fusion was inhibited using N-ethylmaleimide or anti-NSF antibodies (Alvarez-Domínguez et al., 1997; Funato et al., 1997). The requirement for NSF suggests the involvement of SNAREs, of which
both v- and t-SNAREs have been localized to phagosomes (Desjardins et al., 1997; Hackam et al., 1996), which may mediate phagosome fusion with the various endocytic compartments.

The maturation of the phagosome is essential for microbial killing, since microorganisms that avoid maturation survive within the host cell. The intracellular pathogens Mycobacterium and Listeria are found in phagosomes that do not progress past the early stages of maturation. Markers of the early endosomes such as the transferrin receptor and Rab5 accumulate on these phagosomes (Alvarez-Dominguez et al., 1997; Sturgill-Koszycki et al., 1996), whereas late endosomal markers such as LAMP1 and Rab7 do not (Alvarez-Dominguez et al., 1997; Via et al., 1997). It has been suggested that these organisms actively prevent their maturation through the retention and/or accumulation of Rab5 (Alvarez-Dominguez et al., 1997; Via et al., 1997), which would promote docking and fusion with the early endosomes, thus avoiding an interaction with late compartments.

ENGINEERED PHAGOCYTIC CELLS

Phagocytic cells can be engineered from non-phagocytic cells by simply transfecting them with phagocytosis-promoting receptors. For instance, COS cells transfected with FcγRs can internalize IgG-opsonized particles (Indik et al., 1991; Indik et al., 1995). Not only are such engineered phagocytes capable of internalizing opsonized particles, but the resultant phagosomes mature in a manner similar to those in native phagocytes (Downey et al., 1999). The phagosomes formed by Chinese hamster ovary cells stably transfected with FcγRIIA receptors (CHO-IIAs) sequentially acquire the transferrin receptor and LAMP1, indicating that they fuse with the early and eventually the late endosomes and lysosomes. Furthermore, they acidify to the same extent and, most importantly, are able to inhibit the growth of bacteria. There are
several advantages to using engineered rather than native phagocytes. Native phagocytes have many phagocytosis-promoting receptors, including several classes of FcγRs, making it difficult to activate, and thus study the signals generated by, a single type of receptor. Furthermore, they are difficult to transfec. In contrast, it is possible to express and study a single type of receptor in cells such as fibroblasts, which are readily transfected. Engineered phagocytes have been used to study the structural elements of FcγRs that are important for the initiation of phagocytosis, as well as the other proteins that are involved in propagating the process (Indik et al., 1995).
RATIONALE AND HYPOTHESIS

During phagocytosis, if the membrane of the forming phagosome was simply derived from existing plasma membrane, it would result in a decrease in cell surface area and thus limit the amount of particles internalized. However, phagocytes can internalize multiple particles and yet preserve their surface area during this process. Early studies have demonstrated that macrophages can internalize an area equivalent to ~100% of their original membrane, with little or no reduction in exposed membrane (Werb and Cohn, 1972). Furthermore, flow cytometry determinations (Hackam et al., 1998), as well as estimates of plasma membrane area by measurement of electrical capacitance (Holevinsky and Nelson, 1998), revealed that, rather than decreasing, the cell surface often increases during the course of phagocytosis. These findings suggest that exocytosis, i.e. the fusion of endomembranes with the plasma membrane, accompanies phagocytosis. This conclusion is consistent with the net increase in plasma membrane area reported to occur during spreading of macrophages on IgG-coated surfaces, a process analogous to abortive phagocytosis (Cox et al., 1999).

It is not clear if the putative exocytosis of endomembranes occurs at the time of phagocytosis, or whether it is a delayed compensatory response. It is similarly unclear whether exocytosis occurs randomly throughout the cell surface, or if it is instead targeted to the region of the forming phagosome. It has been suggested that exocytosis may be occurring at the time and site of phagocytosis since inhibition of PI3K, which regulates many membrane trafficking events within the cell (Martin, 1998), including those to the plasma membrane (Siddhanta et al., 1998), was shown to inhibit phagocytosis by preventing maximal pseudopod extension (Cox et al., 1999). Finally, the source of the endomembranes required to compensate for the area internalized remains unclear. In this regard, it was recently shown that the microinjection of
TeTx into macrophages causes a decrease in their efficiency of phagocytosis (Hackam et al., 1998). As mentioned in the introduction, TeTx is known to inhibit exocytosis in a variety of systems by proteolyzing certain isoforms of VAMP (Schiavo et al., 1992). We therefore speculated that compartments expressing TeTx-sensitive isoforms of VAMP would be likely to undergo exocytosis during phagosome formation. Among these, VAMP3 is most widely expressed and is predominantly localized to the recycling compartment of the early endosomes (Daro et al., 1996; McMahon et al., 1993). To test this prediction, and to analyze the spatial and temporal pattern of endomembrane delivery, we monitored the distribution of VAMP3 during the course of phagocytosis. For this purpose, we used antibodies raised to the endogenous VAMP3, as well as transfection of a chimeric construct of VAMP3 with GFP (VAMP3-GFP, Fig. 1.5). Activation by a single, well-defined opsonin receptor was ensured by using Chinese hamster ovary cells stably transfected with FcγRIIA receptors (CHO-IIA cells). These cells not only recapitulate the phagocytic sequence (Downey et al., 1999), but are more amenable to transfection than native phagocytes.
Figure 1.5. VAMP3-GFP fusion protein and its orientation in cell membranes. GFP is attached to the C-terminus of VAMP3 such that the GFP tag is lumenal if the fusion protein is localized to an endomembrane compartment, or extracellular if it is localized to the plasma membrane.

PM: plasma membrane.
CHAPTER 2
MATERIALS AND METHODS

Materials and media

G418 sulfate, cytochalasin D, and thapsigargin were from Calbiochem (La Jolla, CA). Human IgG, 0.8 μm blue-dyed latex beads, 3 μm latex beads, and wortmannin were from Sigma (St. Louis, MO). Sheep red blood cells (RBCs) and rabbit anti-RBC IgG were from ICN-Cappel. Cy3-conjugated donkey anti-human IgG F(ab) fragment, anti-mouse IgG, anti-rabbit IgG, FITC-conjugated donkey anti-human IgG, and horseradish peroxidase-conjugated donkey anti-rabbit IgG were all from Jackson ImmunoResearch Laboratories (West Grove, PA). Rabbit anti-GFP IgG, the acetoxymethyl ester of 1,2-bis(aminophenoxy)ethane-N,N,N',N'-tetraacetic acid (BAPTA-AM), FM1-43, and rhodamine phalloidin were from Molecular Probes (Eugene, OR). Mouse anti-LAMP1 antibody was from the Developmental Studies Hybridoma Bank, maintained by the University of Iowa and John Hopkins University School of Medicine (Baltimore, MD).

Dulbecco’s Modified Eagle’s Medium (DMEM) and α-modified Eagle’s medium (α-MEM) were obtained from Cellgro (Herndon, VA). Fetal bovine serum was from GIBCO (Grand Island, NY) and was heat-inactivated by incubation at 60°C for 30 min. Hepes-buffered bicarbonate-free RPMI 1640 medium was from Sigma. Ca²⁺-free medium contained (in mM): 140 NaCl, 5 KCl, 5 glucose, 1 MgCl₂, 1 EGTA, and 20 Hepes (pH 7.4).

Constructs

To generate the VAMP3-GFP fusion protein construct (provided by Dr. Xiao-Rong Peng, The Hospital for Sick Children, Toronto), full-length rat VAMP3 was PCR-amplified from
pCMV5-VAMP3 (provided by Dr. Thomas Sudhof, University of Texas Southwestern Medical Center, Dallas) using the oligonucleotide primers

5'-GAAGATCTCGCCACCACGTCTACAGGGGTGCCTTCAG and
5'-CCCAAGCTTAGAGACACACCACACAATGATG-3'. The cDNA was ligated into the BglII and HindIII sites of the pEGFP-N1 expression vector (Clontech, Palo Alto, CA). The construction of a vector comprising full-length VAMP3 fused to the CH2 and CH3 domains of the human IgG heavy chain (VAMP3-Ig, provided by Dr. Hsiao-Ping Moore, University of California, Berkeley) was described previously in Teter et al. (Teter et al., 1998). A membrane-targeted form of GFP (PM-GFP, provided by Dr. Tobias Meyer, Duke University) was engineered by fusing the NH2-terminal 10–amino acid acylation sequence of Lyn to GFP (Teruel et al., 1999).

**Cell culture and transfection**

Chinese hamster ovary cells stably transfected with FcγRIIA receptors (CHO-IIA, provided by Dr. Alan D. Schreiber, University of Pennsylvania School of Medicine) were maintained in DMEM supplemented with 10% fetal calf serum and 1 mg/ml G418. Chinese hamster lung fibroblasts stably transfected with FcγRIIA receptor tagged at its COOH terminus with enhanced GFP were maintained in α-MEM supplemented with 10% fetal calf serum and 1 mg/ml G418. The murine cell line J774 was maintained in DMEM with 10% fetal calf serum. All cell lines were maintained at 37°C under 5% CO2.

Where indicated, cells grown on 25-mm glass coverslips were transiently transfected with cDNAs encoding either VAMP3-GFP, PM-GFP, or VAMP3-Ig. In all cases the cells were
transfected using the Fugene-6 reagent (Boehringer Mannheim) as suggested by the manufacturer and used 2 days after transfection.

**Phagocytosis**

CHO-IIA cells were incubated at 37°C with either rabbit IgG-opsonized sheep RBC (RBC-Ig) or human IgG-opsonized latex beads (3 µm; Sigma) for 10 min. Excess particles were washed away with phosphate-buffered saline (PBS) and the cells were incubated in DMEM at 37°C for additional times. To identify adherent RBC-Ig or opsonized latex beads that were not internalized, the samples were incubated at 4°C in Hepes-buffered medium containing Cy3-labeled donkey anti-rabbit IgG (1:1,500) or FITC-labeled donkey anti-human IgG (1:1,000) for 40 min.

Where indicated, calcium transients during phagocytosis were precluded by preloading the cells with BAPTA by incubation with 10 µM of the parent acetoxymethyl ester for 20 min at 37°C in Ca²⁺-free medium, followed by depletion of the intracellular stores by addition of 100 nM thapsigargin for 10 min at 37°C. To inhibit PI3K function during phagocytosis, cells were preincubated in serum-free medium containing 200 nM wortmannin for 1 h at 37°C. To disrupt the actin cytoskeleton during phagocytosis, cells were preincubated in serum-free medium containing 10 µM cytochalasin D for 30 minutes at 37°C.

To assess changes in cell surface area during phagocytosis, CHO-IIA cells which had internalized IgG-opsonized latex beads were trypsinized, washed several times with ice-cold PBS, and resuspended at 10⁶ cells/ml. Control cells were not exposed to particles. Cells were then incubated with 1 µM FM1-43 for 5 min on ice to label the cell surface (Betz and Bewick,
1992), and FM1-43 fluorescence was quantified by flow cytometry. Cells containing latex beads were identified by their increased side scatter.

**Fluorescence labeling and analysis**

To identify early endosomes, cells transfected with VAMP3-GFP were serum-starved for 1 h in DMEM, followed by incubation in serum-free DMEM containing 25 μg/ml tetramethylrhodamine-labeled transferrin for 1 h at 37°C. Labeling of surface VAMP3-Ig was accomplished by incubating transfected cells for 40 min at 4°C in Heps-buffered medium containing Cy3-labeled anti-human IgG F(ab) fragment (1:1,000). Extracellular VAMP3-GFP was labeled by incubation at 4°C in Heps-buffered medium containing rabbit anti-GFP (1:10,000) for 40 min, followed by fixation with 4% paraformaldehyde for 1 h and addition of Cy3-conjugated secondary antibody. To visualize F-actin, cells were fixed with 4% paraformaldehyde for 1 h, permeabilized with 0.1% Triton X-100, and labeled with rhodamine phalloidin (1:1,000) for 1 h. To localize the lysosome-associated membrane protein-1 (LAMP1), cells were fixed with -20°C methanol for 15 min, labeled with mouse anti-LAMP1 for 40 min, followed by a Cy3-conjugated secondary antibody. Fluorescence images were acquired on a DMIRB Leica microscope with a Micromax cooled CCD camera (Princeton Instruments, NJ).

**Phagosome isolation**

Phagosomes were isolated by the method described in Desjardins et al. (Desjardins et al., 1994). In brief, J774 cells were grown on 14-cm petri dishes to 80–90% confluence. Blue-dyed latex beads were added and the mixture was incubated at 37°C for 10 or 60 min. The cells were then washed with ice-cold PBS, resuspended in homogenization buffer (250 mM sucrose, 3 mM
imidazole, plus protease inhibitors) and lysed using a Dounce homogenizer. Unbroken cells were removed and the lysate was mixed with a 60% sucrose, 3 mM imidazole stock (pH 7.4) to obtain a 40% sucrose-imidazole solution, which became part of a discontinuous gradient consisting of 10, 25, 35, 40, and 60% sucrose-imidazole steps. The gradient was centrifuged at 100,000 g for 60 min and the phagosome fraction was collected from the 10–25% interphase. After washing in PBS, the protein concentration of the phagosomal preparation was determined using the bicinchoninic acid assay (BCA; Pierce, Rockford, IL), using BSA as a standard.

**SDS-PAGE and immunoblotting**

Samples were solubilized in Laemmli's sample buffer, resolved by SDS-PAGE, and transferred onto polyvinylidene difluoride membranes. Membranes were blocked overnight with 5% milk in PBS and 0.05% Tween 20 and then incubated with affinity-purified rabbit antibodies to EEA1 (1:1,000, provided by Dr. Marino Zerial, European Molecular Biology Laboratory, Heidelberg, Germany), to the 39-kD subunit of the V-ATPase (1:2,000), or to VAMP3 (1:100) for 1 h in PBS-Tween containing 1% serum albumin. The blots were then washed in PBS-Tween, followed by a 1 h incubation with horseradish peroxidase–conjugated donkey anti–rabbit IgG (1:2,500). Finally, the membranes were washed and developed using enhanced chemiluminescence (Amersham, Arlington, IL).
CHAPTER 3
RESULTS

VAMP3 is present in phagocytes and becomes enriched in phagosomal membranes

To determine the TeTx-sensitive isoforms of VAMP expressed in phagocytic cells we examined total cell lysates from two macrophage cell lines, J774 and RAW264.7. Western blotting revealed that these cells expressed comparatively little VAMP2, while VAMP3 was abundantly expressed (Fig. 3.1). Therefore, we confined our studies to the distribution of VAMP3 during phagocytosis.

The available antibodies to VAMP3 were not suitable for analysis of subcellular distribution by immunofluorescence. Instead, we analyzed the association of VAMP3 with phagosomes by a combination of cellular fractionation and immunoblotting. J774 cells were allowed to internalize latex beads, which were subsequently purified by flotation on density gradients (Desjardins et al., 1994). As illustrated in Fig. 3.2, VAMP3 was greatly enriched in the early phagosomal membrane relative to a whole cell lysate. The early endosomal membrane marker EEA1 (Mu et al., 1995), was also detectable in early phagosomes, but was not noticeably enriched compared with the cell lysate. Expectedly, the V-ATPase was also enriched in the phagosome fraction (Fig. 3.2), consistent with earlier findings (Pitt et al., 1992). Two hours after phagocytosis, the V-ATPase remained enriched in the phagosomes, but both VAMP3 and EEA1 had been largely depleted from the phagosomal membrane (Fig. 3.2).

VAMP3-GFP localizes to the recycling endosomes

While the preceding experiments indicate that VAMP3 accumulates in early phagosomes, they provide little information regarding the precise site and time of incorporation
Figure 3.1. Detection of TeTx-sensitive VAMP proteins in macrophage cell lines. Samples of whole cell lysates of J774 and RAW cells were resolved by SDS-PAGE and immunoblotted with polyclonal antibodies against VAMP2 (top) or VAMP3 (bottom). The amount of protein loaded in each lane is indicated at the top.
Figure 3.2. VAMP3 is enriched in early phagosomes. J774 cells were allowed to internalize latex beads for either 20 min or 2 h and phagosomes were purified by gradient centrifugation. Samples of whole cell lysates (WC) or of phagosomes formed after 20 min (Ph20) or 2 h (Ph120) were resolved by SDS-PAGE and immunoblotted with polyclonal antibodies against EEA1 (top), the 39-kD subunit of the V-ATPase (middle), and VAMP3 (bottom). The amount of protein loaded in each lane is indicated at the top.
<table>
<thead>
<tr>
<th>Protein</th>
<th>15 ug WC</th>
<th>5 ug WC</th>
<th>5 ug Ph20</th>
<th>5 ug Ph120</th>
</tr>
</thead>
<tbody>
<tr>
<td>EEA1</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>V-ATPase</td>
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<tr>
<td>VAMP3</td>
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- EEA1: 170 kDa
- V-ATPase: 39 kDa
- VAMP3: 17 kDa
of the v-SNARE into the phagosomal membrane. Because isolation of purified membranes from phagosomes at earlier stages of formation is not feasible, we opted instead to follow the distribution of VAMP3 during particle internalization by noninvasive means, monitoring the fluorescence of GFP. A VAMP3-GFP chimeric construct was transfected into CHO-IIA cells, which were validated earlier as good models for the study of phagosomal formation and maturation (Downey et al., 1999). Unlike phagocytes, these cells are readily transfectable and are comparatively large and flat, facilitating the subcellular localization of the probe.

The distribution of VAMP3-GFP was monitored by fluorescence microscopy and compared with that of rhodamine-labeled transferrin. As shown in Fig. 3.3, a and b, VAMP3-GFP accumulates predominantly in juxtanuclear early endosomes, likely the recycling subcompartment, as described earlier for endogenous VAMP3 (Daro et al., 1996; McMahon et al., 1993). Because VAMP3 is a type II protein, its COOH terminus is expected to reside in the lumen of the endosomes and to be exposed to the extracellular milieu while on the plasma membrane. We took advantage of this feature to ascertain that the heterologously expressed protein is properly oriented and, more importantly, that it is able to recycle between endomembranes and the plasmalemma. CHO-IIA cells were cotransfected with VAMP3-GFP and VAMP3-Ig. To assess the partition of VAMP3 between the plasma membrane and recycling endosomes, cells were exposed to Cy3-labeled antibody against human IgG at 4°C to label only exofacial VAMP3. Monovalent F(ab) fragments of the antibody were used to preclude aggregation and possible mistargetting. It is noteworthy that, while the fraction of plasmalemmal VAMP3 can be readily detected by this method (Fig. 3.3d), the majority of the v-SNARE is located intracellularly. Indeed, when visualized by the fluorescence of GFP in the same cells, the plasmalemmal component of VAMP3 is barely detectable (Fig. 3.3c, see also Fig.
Figure 3.3. Localization and recycling of VAMP3 in CHO-IIA cells. (a and b) CHO-IIA cells transfected transiently with VAMP3-GFP were incubated with 25 μg/ml tetramethylrhodamine-labeled transferrin for 1 h at 37°C, washed and visualized by fluorescence microscopy. (a) VAMP3-GFP; (b) transferrin. (c–f) CHO-IIA cells transiently cotransfected with VAMP3-GFP and VAMP3-Ig were incubated at 4°C with Cy3-labeled F(ab) fragments of antibodies raised against human IgG, to label exofacial VAMP3-Ig. In c and d the cells were visualized at this stage. In e and f, the cells were next warmed to 37°C and incubated for an additional h to allow internalization of the labeled VAMP3-Ig. (c and e) VAMP3-GFP; (d and f) Cy3-labeled VAMP-Ig. Bar, 10 μm.
3.3, a and e). When cells prelabeled with the F(ab) fragments were subsequently rewarmed to 37°C for 1 h to re-initiate membrane traffic, the conjugated VAMP3-Ig was rapidly internalized and its pattern merged with that of VAMP3-GFP (Fig. 3.3, e and f). Jointly, these observations indicate that chimeric forms of VAMP3 target predominantly to early endosomes, where they appear to be competent for recycling.

**Redistribution of VAMP3-GFP during phagocytosis**

The fate of VAMP3 during phagocytosis was analyzed next by transient transfection of CHO-IIA cells. Formation of phagosomes was induced by exposure of the transfectants to RBC-Ig. Cy3-labeled antibodies to the opsonizing IgG were used to assess the location of the particles. As illustrated in Fig. 3.4a, a redistribution of VAMP3-GFP occurred shortly after exposure to RBC-Ig. At early times (10 min) VAMP3 was found to demarcate the periphery of forming phagosomes. The accumulation of VAMP3-GFP around the RBC-Ig appeared to precede internalization of the particles, as indicated by the fact that the RBC-Ig remained accessible to anti-IgG antibodies added extracellularly (Fig. 3.4b). As time proceeded, the particles became fully internalized and therefore inaccessible to the antibody (Fig. 3.4d). Concomitantly, the amount of VAMP3-GFP associated with the phagosome decreased (Fig. 3.4c), becoming undetectable by 90 min (Fig. 3.4, e and f). Together, these observations indicate that VAMP3-containing endomembranes become transiently associated with the phagosome.

**Increased surface exposure of VAMP3 during phagocytosis**

Accumulation of the fluorescent protein in the vicinity of nascent phagosomes indicates mobilization of VAMP3 to the cell periphery, but does not provide evidence that exocytosis
Figure 3.4. Localization of VAMP3-GFP in CHO-IIA cells during phagocytosis. CHO-IIA cells transfected with VAMP3-GFP were incubated at 37°C with RBC-Ig for 10 (a and b), 30 (c and d), or 90 min (e and f). The distribution of VAMP3-GFP is shown in a, c, and e. In b and d, extracellular RBC-Ig were detected by incubation at 4°C with Cy3-labeled antibodies against the opsonizing IgG. f shows the differential interference contrast image of the cell in e. Arrowheads indicate internalized RBC-Ig. Bar, 10 μm.
occurred at this site. To evaluate this possibility, we monitored the extracellular appearance of the COOH terminus of the chimera using an antibody against GFP. CHO-IIA cells transiently transfected with VAMP3-GFP were allowed to interact with opsonized particles for 10 min and, after cooling to 4°C, the exposure of GFP to externally added primary and secondary antibodies was examined. As before, VAMP3-GFP accumulated around forming phagosomes (Fig. 3.5a). Importantly, the COOH-terminal domain of the chimera was exposed to the external medium, as attested by its accessibility to anti-GFP antibodies (Fig. 3.5b). Note that under these conditions the remaining juxtanuclear VAMP3-GFP remains inaccessible to the antibody.

The marked enrichment of VAMP3-GFP in the area of the plasmalemma subjacent to the opsonized particle suggests that focal exocytosis of recycling endosomes occurred at the site of phagosome formation. However, because a fraction of the VAMP3 is present constitutively at the plasma membrane, accumulation underneath the phagosome could also have occurred as a result of lateral diffusion and focal immobilization of the chimera. Indeed, as mentioned in the Introduction, such lateral mobilization or capping is responsible for the enrichment of Fc receptors in the phagosomal cup. We therefore compared the lateral displacement of FcγRIIA receptors and of plasmalemmal VAMP3 during the course of particle binding and cup formation. Chinese hamster cells were co-transfected with a GFP-tagged FcγRIIA receptor and with VAMP3-Ig. The exofacial VAMP3-Ig was then labeled with Cy3-conjugated F(ab) fragments at 4°C before phagocytosis. Under these conditions, both the FcRIIA receptors and the labeled VAMP3-Ig are homogeneously distributed on the plasma membrane (not illustrated). Upon exposure to opsonized particles, accumulation of FcRIIA receptors in phagosomal cups was readily apparent (Fig. 3.5c). In contrast, no accumulation of the prelabeled VAMP3 was detected under these conditions (Fig. 3.5d). These findings suggest that the concentration of
Figure 3.5. Surface exposure of VAMP3-GFP in CHO-IIA cells during phagocytosis. (a–b) CHO-IIA cells transfected with VAMP3-GFP were incubated at 37°C with IgG-opsonized latex beads for 10 min. After stopping phagocytosis by cooling to 4°C, exofacial VAMP3-GFP was detected by incubation with antibodies to GFP followed by secondary Cy3-conjugated antibodies. (a) Distribution of VAMP3-GFP. Arrows indicate beads that are undergoing phagocytosis, whereas the arrowhead indicates a bead not being internalized. Inset shows the corresponding differential interference contrast image. (b) Distribution of exofacial VAMP3-GFP. (c and d) Cells stably expressing FcγRIIA-GFP were transiently transfected with VAMP3-Ig and then incubated at 4°C with Cy3-labeled F(ab) fragments of antibodies raised against human IgG, to label exofacial VAMP3-Ig. Finally, opsonized RBC were added to induce receptor clustering. (c) Distribution of FcγRIIA-GFP. Arrowheads point to FcγRIIA-GFP clusters formed at sites of interaction with the RBC. (d) Distribution of VAMP3-Ig. (e and f) CHO-IIA cells were transiently transfected with PM-GFP. Next, RBC-Ig were added to initiate phagosome formation. (e) Differential interference contrast image. Arrowhead indicates a forming phagosome. (f) Distribution of PM-GFP in the cell shown in (e). Bar, 10 μm.
exofacial VAMP3-GFP in the vicinity of the phagosome reported in Figs. 3.4 and 3.5 is not likely due to lateral accumulation of molecules present at the surface before phagocytosis.

It may be argued that rather than resulting from the targeted exocytosis of endomembranes, the enhanced VAMP3-GFP signal at nascent phagosomes merely reflects an increase in the overall membrane density in the region of the ingested particle, perhaps resulting from localized ruffling. To examine this possibility, we monitored the fluorescence of PM-GFP. Upon acylation, promoted by attachment of the 10 NH2-terminal amino acids of Lyn (Teruel et al., 1999), GFP acquires the tendency to bind predominantly to the inner leaflet of the plasmalemma (Fig. 3.5f). In cells exposed to RBC-Ig, PM-GFP followed the contour of the phagosomal cup, but failed to show the marked accumulation reported above for VAMP3-GFP. Thus, localized ruffling of the plasmalemma cannot explain the accumulation of VAMP3 in the region of the nascent phagosome.

Change in cell surface area during phagocytosis

To further verify that the exocytosis of endomembranes accompanies phagocytosis, we examined the change in cell surface area during particle ingestion. CHO-IIA cells were initially allowed to ingest opsonized latex beads. Under the conditions used, each of the phagocytically active cells ingested five or more beads. Considering the size of the latex beads used (3 μm in diameter) an area equivalent to 27% of the original surface area of CHO-IIA cells was internalized (calculated assuming 5 beads/cell and a cell surface area of ~530 μm², based on a spherical cell diameter of 13 μm). To estimate the effective change in surface area accompanying this ingestion, we used the dye FM1-43, which becomes fluorescent when intercalated into the outer leaflet of the plasma membrane (Betz and Bewick, 1992). Cells with
and without internalized particles were detached, cooled on ice and incubated with FM1-43. Flow cytometry analysis of FM1-43 fluorescence revealed that cells with internalized particles, identified by the increase in their side scatter relative to control cells, had an average fluorescence increase of ~35%. The enhanced fluorescence, which reflects increased surface area after phagocytosis, further supports the notion that endomembranes are inserted into the plasmalemma during this process, consistent with the appearance of VAMP3 at sites of phagosome formation.

**VAMP3 accumulation does not require calcium**

Cross-linking of Fc receptors has been shown to increase free cytosolic calcium in phagocytes (Di Virgilio et al., 1988; Young et al., 1984). In addition, elevation of cytosolic calcium upon addition of ionophores was shown earlier to accelerate the exocytosis of endosomes in murine macrophages (Buys et al., 1984). It was therefore conceivable that localized changes in calcium triggered the focal exocytosis of VAMP3-containing membranes during FcR-mediated phagocytosis. To test this notion, CHO-IIA cells were loaded with BAPTA and pre-treated with thapsigargin in calcium-free medium, in order to deplete the calcium stored in the ER. When subsequently exposed to opsonized particles, the localized accumulation of VAMP3-GFP in the vicinity of nascent phagosomes was unaffected (data not shown). These findings are in accordance with the observation that, in macrophages, phagocytosis is calcium independent (Di Virgilio et al., 1988; Greenberg et al., 1991).

**VAMP3 accumulation is sensitive to wortmannin and cytochalasin**

The trafficking of recycling endosomes to the plasma membrane is thought to be regulated by one or more products of PI3Ks (Siddhanta et al., 1998). This conclusion was
reached on the basis of the inhibitory effects of wortmannin (Martys et al., 1996) and class-specific antibodies (Siddhanta et al., 1998). Importantly, wortmannin prevents completion of phagocytosis (Araki et al., 1996; Ninomiya et al., 1994), apparently by precluding the extension of pseudopods around the opsonized particle (Cox et al., 1999). We therefore tested the effects of wortmannin on VAMP3-GFP redistribution. As reported earlier for native phagocytes, pretreatment of CHO-IIA cells with the PI3K inhibitor prevented phagocytosis, without affecting particle binding (Fig. 3.6a) or the polymerization of actin under the phagocytic cup (Fig. 3.6b) (Araki et al., 1996; Cox et al., 1999; Ninomiya et al., 1994). More importantly, wortmannin eliminated the accumulation of VAMP3-GFP near the attached particles in most cells (Fig. 3.6c). The VAMP3-GFP-containing endomembranes of these cells appeared enlarged relative to those of untreated cells, implying that the trafficking of membranes from the compartment was affected. The impaired delivery of VAMP3-containing endomembranes to the plasma membrane may therefore account for the inhibitory effect of wortmannin on pseudopod extension.

F-actin accumulates in forming phagocytic cups (Allen and Aderem, 1996; Greenberg et al., 1990) and this polymerization is essential since cytochalasins (Zigmond and Hirsch, 1972), drugs which prevent actin polymerization, inhibit phagocytosis. These findings have prompted the suggestion that the force generated through actin polymerization may drive the extension of plasma membrane, which we have now found to be at least partially derived from VAMP3-containing endomembranes, over particles. We therefore wanted to analyze the effects of a cytochalasin on VAMP3-GFP redistribution during phagocytosis. CHO-IIA cells pretreated with cytochalasin D were unable to phagocytose particles as previously reported (Zigmond and Hirsch, 1972), though binding was unaffected (Fig. 3.7a). The actin cytoskeleton of these cells
Figure 3.6. Effect of wortmannin on VAMP3-GFP redistribution during phagocytosis. CHO-IIA cells were incubated at 37°C for 1 h in medium containing 200 nM wortmannin, followed by a 30 min incubation with RBC-Ig. (a) Differential interference contrast image. (b) F-actin staining with rhodamine-phalloidin. (c) VAMP3-GFP distribution. Arrowheads indicate phagocytic cups. Bar, 10 μm.
Figure 3.7. Effect of cytochalasin on VAMP3-GFP redistribution during phagocytosis. CHO-IIA cells were incubated at 37°C for 30 min in medium containing 10 μM cytochalasin, followed by a 20 min incubation with RBC-Ig. (a) Differential interference contrast image. (b) F-actin staining with rhodamine-phalloidin. (c) VAMP3-GFP distribution. Arrowheads indicate phagocytic cups. Bar, 10 μm.
was disrupted (Fig. 3.7b, compare with the intact actin cytoskeleton in Fig. 3.6b), and both F-actin (Fig. 3.7b) and VAMP3-GFP (Fig. 3.7c) failed to accumulate at sites of particle binding. It is possible that actin polymerization at sites of particle binding is necessary to structure exocytosed VAMP3-containing endomembranes around the particle. Therefore, an enhanced VAMP3-GFP signal was not detectable in cytochalasin-treated cells possibly because the exocytosed VAMP3-GFP had diffused along the plane of the membrane by the time we visualized them. Alternatively, vesicles translocating to sites of exocytosis at the plasma membrane may do so on the actin cytoskeleton via myosin motor proteins (Mermall et al., 1998), and disruption of the actin cytoskeleton would inhibit their translocation to, and thus exocytosis at, sites of particle binding. However, it cannot be ruled out that phagocytosis was inhibited at a stage prior to exocytosis and even vesicle delivery, such as at the initial stage of receptor clustering. As mentioned in the introduction, it seems that an intact actin cytoskeleton is essential for receptor clustering since inhibition of the Rho GTPase decreases the F-actin content of macrophages and inhibits phagocytosis at the stage of receptor clustering (Hackam et al., 1997). Clearly, further studies will have to be done in order to address the stage at which phagocytosis is affected by cytochalasin treatment.

**Distribution of LAMP1 during phagocytosis**

Recruitment and exocytosis of lysosomes was shown to be required for *Trypanosoma cruzi* invasion of mammalian cells (Tardieux et al., 1992). To evaluate whether lysosomes are also secreted in the vicinity of the forming phagosome, we analyzed the distribution of LAMP1, a late endosomal/lysosomal marker. Unlike the early and transient interaction found above for VAMP3, the LAMP1-containing compartment was not mobilized to the area of the nascent
phagosome (Fig. 3.8, a and b). Instead, LAMP1-containing vesicles merged with the phagosome only at later stages of maturation, becoming clearly detectable after 60 min (Fig. 3.8, c and d). Therefore, insertion of LAMP1-containing vesicles is unlikely to contribute significantly to the increase in surface area observed upon phagocytosis.
Figure 3.8. Localization of LAMP1 in CHO-IIA cells during phagocytosis. CHO-IIA cells were incubated at 37°C with IgG-opsonized latex beads for 10 min (a and b) or 60 min (c and d). The distribution of LAMP1, detected by indirect immunofluorescence using Cy3-labeled antibodies, is shown in a and c. b and d show the differential interference contrast images of the cells in a and c, respectively. In a and b, arrowheads indicate beads that have been internalized, whereas arrows indicate extracellular beads, identified by accessibility to extracellularly added labeled antibodies. (Inset) Extracellular particles were detected by incubation at 4°C with FITC-labeled antibodies against the opsonizing IgG. Bar, 10 μm (does not apply to inset).
CHAPTER 4
DISCUSSION AND FUTURE DIRECTIONS

Membrane dynamics during phagosome formation

It was previously shown that phagocytosis by J774 and CHOIIA cells could be partially inhibited by microinjection or transfection of TeTx (Hackam et al., 1998), suggesting that a VAMP dependent vesicle fusion step is important for phagosome formation. In this study we have shown that the TeTx substrate VAMP3 is targeted to the nascent phagosome, where it is secreted focally before closure of the phagosomal membrane. VAMP3 is a primary component of the recycling endocytic compartment and has been implicated in the delivery of transferrin receptors to the cell surface (Galli et al., 1994). Early endosomes had been shown earlier to deliver transferrin receptors to formed phagosomes during the course of their maturation (Sturgill-Koszycki et al., 1996). Our studies indicate that vesicles derived from the recycling compartment are important not only in the maturation of phagosomes, but also in their formation.

The concept that membrane addition may be required for phagocytosis has only recently come about, as previous models had proposed that the internalization of particles resulted from the direct apposition of the cell membrane to the surface of the particle via opsonin receptors by a zippering action (Griffin et al., 1975). This hypothesis would predict a net loss of cell surface area during phagocytosis, particularly when large and/or multiple particles are ingested. In actuality, electrophysiological studies have demonstrated a transient but rapid increase in membrane capacitance during the signaling phase of phagocytosis, followed by stepwise decreases that correspond with particle internalization (Holevinsky and Nelson, 1998). So, although the accumulation of F-actin around the nascent phagosome has led many to speculate
that the growth of pseudopods around the particle is driven by the extension of actin filaments (Greenberg et al., 1990), it may be mediated, at least in part, by the focal delivery of endomembranes to the area of phagosome formation (Fig. 4.1). The increase in surface area would permit the receptor- and/or actin cytoskeleton-driven zippering of the membrane around the particle. Therefore, we regard endomembrane insertion not as an alternative but as complementary to the zippering model.

**Trafficking components involved in membrane delivery**

The v-SNARE VAMP3 is likely essential for the fusion of VAMP3-containing vesicles with the plasma membrane. However, in addition to VAMP3, there are probably many other proteins involved in the targeting and fusion of these vesicles with the plasmalemma. A v-SNARE must interact with t-SNAREs in order to mediate membrane fusion. The t-SNAREs that may potentially interact with VAMP3 include syntaxin4 and SNAP-23. Syntaxin4 has been localized to the plasma membrane in the macrophage cell line J774, and is also found on the membranes of phagosomes isolated from these cells (Hackam et al., 1996). SNAP-23 is a ubiquitously expressed SNAP-25 isoform, and is present in phagocytic cells (Ravichandran et al., 1996). Targeting of the VAMP3-vesicles to sites of fusion on the plasma membrane is likely mediated by Rab11. It is localized to the recycling endosomes in various cells types, including macrophages, and regulates transferrin recycling in these cells since Rab11 mutants that are unable to bind GTP decrease the rate of transferrin recycling in all of these cell types (Cox et al., 2000; Ullrich et al., 1996). The potential involvement of Rab11 in the targeting of recycling vesicles to the plasma membrane implies that a perturbation of its function should similarly affect phagocytosis as does proteolysis of VAMP3 by TeTx. Indeed, the transfection of J774
Figure 4.1. Focal exocytosis of VAMP3-containing vesicles at sites of phagosome formation.

The clustering of Fc receptors by opsonized particles triggers localized exocytosis of VAMP3-containing vesicles, which may serve to provide membrane for the growth of the pseudopods that ultimately engulf the particle.
sorting endosomes

VAMP3-containing recycling endosomes

nucleus
macrophage cells with Rab11 GTP-binding mutants alters their phagocytosis efficiency (Cox et al., 2000). As with the other Rab proteins, Rab11 may associate with effectors that mediate their association and migration on the cytoskeleton and that serve to dock these vesicles at the plasma membrane.

**ARF6**

The exocytosis of VAMP3-containing vesicles at sites of phagosome formation may not only be necessary for the extension of pseudopods through membrane addition, but also for the delivery of proteins that are necessary for efficient phagocytosis to occur, such as ARF6.

The ARF family of small GTPases consists of several distinct members, with ARF1 being the best characterized (Chavrier and Goud, 1999). The GDP-bound form of ARF1 is cytosolic, whereas the GTP-bound form is localized to the Golgi, this association being dependent on its myristoylation. ARF1 is an essential component in the formation of the coat complex that is involved in the budding of vesicles from the Golgi. The GDP-bound form of ARF6 colocalizes with VAMP3-containing recycling endosomes in cells, whereas the GTP-bound form is localized to the plasma membrane (D'Souza-Schorey et al., 1998). Nucleotide exchange on ARF6 is catalyzed by the cytohesin-1/ARNO/GRP1 family of ARF GEFs, which have a Sec7 domain encoding GEF activity and a PH domain that binds to PIP3, allowing their localization and/or activity to be regulated by PI3K (Jackson and Casanova, 2000). It has been proposed that ARF6 couples the trafficking of endomembranes to the plasma membrane, presumably those of the recycling compartment, with actin polymerization since a constitutively active ARF which is unable to hydrolyze GTP induces actin-rich ruffles on the plasma membrane (Radhakrishna and Donaldson, 1997). Moreover, it has recently been found that
ARF6-GTP binds to and increases the activity of PI4P5K (Honda et al., 1999), which catalyzes the conversion of PIP to PIP2. PIP2, as previously mentioned, promotes actin polymerization by sequestering PIP2-binding proteins such as gelsolin that function to prevent actin filament elongation. It has therefore been suggested that as a recycling endosome containing ARF6-GDP is targeted to the plasma membrane, a GEF promotes its conversion to ARF6-GTP, which serves to localize and activate PI4P5K, leading to actin rearrangements (Venkateswarlu and Cullen, 2000).

In addition to providing membrane to extend the pseudopods, VAMP3-containing recycling vesicles may also contribute to actin polymerization at the plasma membrane, which may serve to drive the extension of the pseudopods, through the delivery of ARF6. PI3K is in fact activated during phagocytosis (Ninomiya et al., 1994), and the resulting increase in PIP3 from resting levels of PIP2 in the plasma membrane could serve to localize ARF6 GEFs through their PIP3-binding PH domain. This would activate ARF6 delivered by VAMP3-containing vesicles, which would induce actin rearrangements by increasing PIP2 levels. Some of PIP2 produced may be converted by PI3K to PIP3, leading to further ARF6 activation, generating a positive-feedback loop. In support of a role of ARF6 in phagocytosis, ARF6 mutants that are unable to bind GTP decrease the efficiency of phagocytosis, with the sites of particle-binding exhibiting poor F-actin accumulation (Zhang et al., 1998).

**Mechanism of membrane accumulation at sites of phagosome formation**

During receptor-mediated endocytosis, surface receptors and their associated ligands are internalized. In order to compensate for the receptors internalized, and to maintain plasma membrane homeostasis, this endocytosis is balanced by the exocytosis of recycling vesicles at
the plasma membrane, which is mediated by VAMP3. At sites of phagosome formation, there are two potential ways in which VAMP3-containing membranes could accumulate to result in an increase in membrane area that ultimately engulfs the particle. Receptor clustering could either be triggering a localized decrease in endocytosis or a localized increase in exocytosis. The following sections will address each of these possibilities.

**Local decrease in endocytosis**

A local decrease in endocytosis at the cell surface could be detected in the following way: The cell surface would have to be uniformly labelled with a protein that would be endocytosed, and the rate of removal of this protein would be monitored over the surface of the cell during phagocytosis. In order to prevent confounding results from recycling, it would be best if the labelled protein could not be routed back to the surface. For instance, the cells could be labelled with low density lipoprotein (LDL) which binds to LDL receptors. LDL is ideal because it dissociates from the receptor in the sorting endosomes and is subsequently routed to degradative compartments (Davis et al., 1987), thus preventing it from resurfacing if internalized. The phagocytes could be placed at 4°C to prevent any membrane trafficking prior to phagocytosis, their surface could be uniformly labelled by incubation in LDL, and particles could then be allowed to bind. Endocytosis of the bound LDL receptors and phagosome formation could be then be induced by incubation at 37°C. Following placement again at 4°C, LDL-receptor complexes remaining on the surface could be detected by using an antibody against the protein portion of LDL. If endocytosis is in fact decreased at sites of phagosome formation, there would be an enhanced apolipoprotein signal at these sites relative to others on the plasma membrane.
However, a localized decrease in endocytosis is not consistent with the fact that amphiphysin (Gold et al., 2000), dynamin (Gold et al., 1999), and clathrin (Aggeler and Werb, 1982) have all been localized to sites of phagosome formation. Furthermore, clathrin-coated pits have been visualized pinching off the plasma membrane of forming phagosomes (Aggeler and Werb, 1982). All of these findings suggest that endocytosis is still actively occurring at these sites. Therefore, it is more likely that there is a local increase in exocytosis at sites of phagosome formation.

**Local increase in exocytosis**

In order to detect a localized increase in exocytosis at sites of phagosome formation, one must be able to distinguish a recycling protein that is secreted from that which is already localized at the membrane. This could be done by constructing a chimera of VAMP3-GFP that is joined together with a thrombin cleavage site. VAMP3-GFP on the surface of the cell could be cleaved with thrombin in order to distinguish it from VAMP3-GFP in the recycling endosomes. Phagocytes could be placed at 4°C to prevent membrane trafficking, VAMP3-GFP on the surface could be cleaved by incubation with thrombin, and particles could then be allowed to bind. Exocytosis of VAMP3-GFP and phagosome formation could then be induced by incubation at 37°C. Following placement again at 4°C, secreted VAMP3-GFP could be detected by using an antibody against GFP. If exocytosis is in fact increased at sites of phagosome formation, there would be an enhanced GFP signal at these sites relative to others on the plasma membrane.

Exocytosis could be locally increased in a number of ways. As discussed in the introduction, it is thought that vesicles are targeted to their final destination through vesicle-
bound Rabs that interact with docking proteins/complexes on the target membrane. The Exocyst or Sec6/8 complex is a docking complex that is localized to the plasma membrane in yeast and epithelial cells and is responsible for targeting vesicles derived from the Golgi (Hsu et al., 1999). Rabs bound to these Golgi-derived vesicles in yeast can directly interact with components of the docking protein/complex (Guo et al., 1999). Recycling vesicles may similarly be targeted to the plasma membrane through such docking complexes that would potentially interact with the Rab11 that is localized to these vesicles. If such docking complexes are involved, it is conceivable that the exocytosis of recycling vesicles is regulated by their abundance on the plasma membrane. Exocytosis could therefore be upregulated at sites of phagosome formation by increasing the amount of docking complexes. To test such a hypothesis would be difficult at this point in time as few effectors, including potential docking factors, of Rab11 have been identified.

The exocytosis of recycling vesicles may be regulated by SNARE accessibility. Proteins exist that bind directly to the SNAREs, referred to as SNARE regulators, and include the nSec1/munc18 family in mammalian cells that bind to syntaxin. Their binding to SNAREs prevents SNARE complex formation and thus negatively regulates membrane fusion (Halachmi and Lev, 1996). The SNAREs involved in the exocytosis of recycling vesicles may be regulated by such proteins and therefore it is possible that an enhancement in their release could increase exocytosis at sites of phagosome formation. The mechanism of release of these proteins is not well known, although the involvement of Rabs has been proposed (Lupashin and Waters, 1997). It is quite possible that there are multiple ways in which these proteins can be regulated. Many proteins are regulated through phosphorylation, and SNARE regulators may be included in this
category. In fact, the serine/threonine protein kinase C (PKC) can phosphorylate nSec1/munc18 (Fujita et al., 1996).

There are several groups of PKC isoforms, based on their regulation (Nishizuka, 1992). Classical PKCs contain both a C1 and a C2 domain that bind to DAG and calcium, respectively, whereas novel PKCs only contain the C1 domain, and thus are regulated only by DAG. The classical isoform PKCα phosphorylates nSec1/munc18, and the resulting phosphorylation inhibits its interaction with syntaxin (Fujita et al., 1996). At sites of phagosome formation, PKC activation could bypass the normal regulation of nSec1/munc18 release from syntaxin, enhancing the amount of recycling vesicles fusing with the plasma membrane. PKC may phosphorylate the nSec1/munc18 homologue that binds to the syntaxin involved in this fusion step, thus inhibiting nSec1/munc18 interaction with syntaxin and allowing syntaxin to interact with VAMP3, leading to vesicle fusion. In support of this, PKCs are activated during phagocytosis (Zhelezyak and Brown, 1992). Both PKCα (Allen and Aderem, 1996; Allen and Aderem, 1995) and the novel isoform PKCδ (Brumell et al., 1999) have been localized to the phagosome. PKC activity appears essential for phagocytosis as inhibitors such as staurosporine and H7 inhibit it (Karimi and Lennartz, 1995; Zhelezyak and Brown, 1992). Furthermore, treatment of macrophages with PMA, an analogue of DAG and thus an activator of PKC, causes massive spreading, which is a result of exocytosis of endomembranes (Buys et al., 1984). This spreading is associated with an increase in transferrin receptor on the surface of the cell, implying that recycling endosomes are the vesicles that are being secreted, and that their exocytosis is regulated by PKC (Buys et al., 1984).

To begin addressing the mechanism proposed above, it would first be necessary to identify all of the SNAREs and their regulators involved in recycling vesicle fusion at the plasma
membrane, such as the syntaxin isoform involved and its cognate nSec1/munc18 homologue. The phosphorylation status of each of these could then be examined during phagocytosis. The phosphorylation of the SNAREs themselves (Shimazaki et al., 1996), and a subsequent effect on exocytosis, cannot be excluded as they too are capable of being phosphorylated.

**Role of microtubules**

Microtubules, polymers of the proteins α- and β- tubulin, are dispersed throughout the cytoplasm of a cell and arise from a common microtubule organizing center (MTOC) which has a juxtanuclear localization. Microtubules are polar: the minus end of a microtubule is that which is located at the MTOC, and the positive end, where polymerization occurs, is oriented towards the plasma membrane. One of the many functions of the microtubule cytoskeleton is to maintain organelles at, or translocate them towards, specific locations in the cell (Hirokawa, 1998). This is achieved through organelle association with motor proteins that bind to microtubules and use the energy of ATP to translocate along them. There are two types of motor proteins: dyneins translocate organelles towards the minus end of microtubules, whereas kinesins translocate organelles towards the plus end (Hirokawa, 1998). The juxtanuclear positioning of the Golgi and the recycling endosomes is dependent on an intact microtubule network since the treatment of cells with the microtubule-disrupting agent nocodazole disperses these organelles throughout the cytoplasm (Yamashiro et al., 1984). At least in the case of the Golgi, this dependence is due to its association with dyneins (Harada et al., 1998) which maintains its position at the MTOC. Vesicles emerging from either the Golgi or recycling endosomes are likely associated with kinesins which would allow their translocation along the microtubule cytoskeleton towards the plasma membrane.
The localization and/or stabilization of microtubules at sites on the plasma membrane may serve to target vesicles derived from the above mentioned organelles. This is indeed known to occur in a number of cell types. During axonal outgrowth in neurons, the microtubule network is polarized toward these sites and serves to target the delivery of membrane vesicles from the Golgi. Similarly, in epithelial cells, the microtubule network is polarized towards the apical domain and serves to localize the delivery of vesicles from the Golgi to this domain. Finally, when helper T-cells interact with antigen-presenting cells (APCs), lymphokines secreted from the Golgi of the helper T-cell are targeted towards the APC (Kupfer et al., 1991) due to the polarization of the helper T-cell microtubule network (Kupfer et al., 1986).

In all of the above mentioned cases, vesicles emerging from the Golgi are targeted to particular sites on the plasma membrane through microtubules. The signalling pathway responsible for the stabilization of microtubules at these sites is not well known. However, at least during helper T-cell-APC interaction, the polarization of the microtubule network has been found to be dependent on the Rho family GTPase Cdc42, since transfection of cells with a GTP-binding mutant of Cdc42 inhibits polarization (Stowers et al., 1995). Cdc42 may be mediating this effect on the microtubule network indirectly through actin reorganization, which was also affected in the cells expressing the Cdc42 mutant. There are a number of known actin-binding proteins that can also bind to microtubules (Fujii et al., 1997; Gonzalez et al., 1998) and thus actin reorganization may potentially establish sites where these proteins can bind and possibly stabilize microtubules.

As with Golgi-derived vesicles, recycling vesicles may be similarly targeted by microtubules. During phagocytosis, microtubules may be stabilized/accumulate at sites of phagosome formation and this may serve to target the recycling vesicles (Fig. 4.2). In order to
Figure 4.2. Hypothetical model of microtubule-dependent VAMP3-containing vesicle targeting to sites of phagosome formation. Accumulation of microtubules at sites of phagosome formation may serve to localize VAMP3-containing vesicles.
address this hypothesis, the importance of microtubules in phagocytosis would have to be
determined using microtubule-disrupting agents such as nocodazole. If microtubules are
necessary, a GFP-tagged microtubule component such as β-tubulin could potentially be useful in
time-lapse imaging experiments to determine whether microtubules are indeed focused at sites of
phagosome formation. If so, the signals responsible for this could be investigated. Cdc42 is
essential for the polarization of microtubules in helper T-cells (Stowers et al., 1995). Phagocytic
cells transfected with a dominant-negative Cdc42 that is unable to hydrolyze GTP are unable to
internalize particles. Interestingly, in these transfectants there was actin accumulation at the
sites of aborted phagocytosis, but no apparent pseudopod extension (Massol et al., 1998). This
finding suggests that Cdc42 may be necessary for the targeting of vesicles, possibly through an
effect on microtubules.

**Other cellular processes potentially mediated by focalized VAMP3 delivery**

Our observation that phagocytosis is mediated by the localized growth of membranes
through targeted delivery of recycling endosomes may have broader implications. Many cellular
processes, such as the formation of lamellipodia, macropinocytosis, cell spreading and
chemotaxis, as well as bacterial invasion, all result from receptor signaling that leads to localized
expansion of the plasma membrane. While actin polymerization has been implicated in each of
these processes, growing evidence supports the notion that membrane addition may also be
required (Bretscher, 1996). The recycling endosome compartment may provide an ideal source
for such localized growth; as it represents a large reservoir of membrane area, it contains the
targeting elements required for fusion with the plasma membrane and is also capable of
interacting with microtubules. Vectorial traffic along microtubules may contribute to the
polarized delivery to the site of receptor activation. Fluorescent forms of VAMP3 should prove useful in testing the role of recycling endosomes in other biological responses.
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