SECOND MESSENGER-MEDIATED REGULATION OF AUTOPHAGY

by

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A thesis submitted in conformity with the requirements for the degree of Doctor of Philosophy
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Second Messenger-mediated Regulation of Autophagy

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ABSTRACT

Autophagy is an evolutionarily conserved degradative eukaryotic cell pathway that plays a role in multiple cellular processes. One important function is as a key component of the cellular immune response to invading microbes. Autophagy has been found to directly target and degrade multiple intracellular bacterial species. In this thesis, I identify and characterize two distinct regulatory mechanisms for this pathway involving the second messengers: diacylglycerol and cyclic adenosine monophosphate (cAMP).

Salmonella enteric serovar Typhimurium (S. Typhimurium) is a Gram-negative bacterial species that has been shown to be intracellularly targeted for degradation by autophagy. While targeting of this species has been previously shown to involve ubiquitination, this pathway accounts for only half of targeted bacteria. Here I show that ubiquitin-independent autophagy of S. Typhimurium requires the lipid second messenger diacylglycerol. Diacylglycerol localization to the bacteria precedes autophagy and functions as a signal to recruit the δ isoform of protein kinase C (PKC) in order to promote the specific autophagy of tagged bacteria. Furthermore, I have found that the role of diacylglycerol and PKCδ is not limited to antibacterial autophagy but also functions in rapamycin-induced autophagy indicating a general role for these components in this process.
Multiple bacterial species have been found to be targeted by autophagy and while some have developed strategies that allow them to avoid targeting, no bacterial factor has yet been identified that is able to inhibit the initiation of this process. Here I show that two bacterial species, _Bacillus anthracis_ and _Vibrio cholera_ inhibit autophagy through the elevation of intracellular cAMP and activation of protein kinase A. Using two different bacterial cAMP-elevating toxins, I show that multiple types of autophagy are inhibited in the presence of these toxins. This is indicative of a general inhibitory function for these toxins and identifies a novel bacterial defence strategy.

This work characterizes both a novel regulatory signal for the induction of autophagy and identifies a novel bacterial tactic to inhibit this process. Together the data presented in this thesis provide novel insight into the regulation of autophagy and offer potential targets for modulation of this process.
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LIST OF ABBREVIATIONS

3-MA: 3-methyladenine
AC: adenylate cyclase
AKAP: A-kinase anchoring protein
AP: autophagosome
ATP: adenosine triphosphate
cAMP: cyclic adenosine monophosphate
CM: chloramphenicol
CMA: chaperone-mediated autophagy
CNGC: cyclic nucleotide-gated ion channel
CREB: cAMP response element binding
Ctx: cholera toxin
DAG: diacylglycerol
DGK: diacylglycerol kinase
Edtx: edema toxin
EF: edema factor
EPAC: exchange protein directly activated by cAMP
ER: endoplasmic reticulum
ERK: extracellular signal-regulated kinase
ETEC: Enterotoxigenic Escherichia coli
FcγR: Fcγ receptor
FCV: Francisella-containing vacuoles
FIP200: focal adhesion kinase family interacting protein of 200 kD
Gα: G protein α subunit
GcAVs: Group A Streptococcus-containing autophagosome-like vacuoles
GDP: guanosine diphosphate
GPCR: G protein-coupled receptor
GTP: guanosine triphosphate
HLtx: heat-labile enterotoxin
Hsc70: heat shock cognate protein of 70 kDa
hVps34: human vacuolar protein sorting 34
IKK: inhibitor of kB kinases
JNK: c-Jun N-terminal kinase
KO: knockout
LAMP1: lysosome-associated membrane protein 1
LAMP-2A: lysosome-associated membrane protein type 2A
LC3: microtubule-associated protein 1 light chain 3
LF: lethal factor
LIR: LC3-interaction region
LLO: listeriolysin O
LPS: lipopolysaccharide
Ltx: lethal toxin
mAtg9: mammalian Atg9
MEF: mouse embryonic fibroblast
MEK: mitogen-activated protein kinase kinase
MIPA: micropexophagy-specific membrane apparatus
mTOR: mammalian target of rapamycin
NBR1: neighbour of BRCA1 gene 1
NDP52: nuclear dot protein 52
NF-κB: nuclear factor -κB
NLR: NOD-like receptor
NOD: nucleotide-binding oligomerization domain
NOX: NADPH oxidase
PA: phosphatidic acid
PC: phosphatidylcholine
PE: phosphatidylethanolamine
Ptx: Pertussis toxin
Pi: phosphatidylinositol
PrA: protective antigen
PS: phosphatidylserine
PAMP: pathogen-associated molecular pattern
PAP: phosphatidic acid phosphatase
PAS: phagophore assembly site
PBMC: peripheral blood mononuclear cell
PDE: phosphodiesterase
PDK1: phosphoinositide-dependent protein kinase 1
PE: phosphatidylethanolamine
PI3K: phosphatidylinositol 3-kinase
PI3P: phosphatidylinositol 3-phosphate
PI4P: phosphatidylinositol 4-phosphate
PKA: protein kinase A
PKC: protein kinase C
PKD: protein kinase D
PLC: phospholipase C
PM: plasma membrane
PRR: pattern recognition receptor
Rlα: regulatory subunit 1-α
RACK: receptors for activated C kinase
ROS: reactive oxygen species
Rubicon: RUN domain and cysteine-rich domain containing, Beclin 1–interacting protein
S1P: sphingosine 1-phosphate
SCV: *Salmonella*-containing vacuole
siRNA: small interfering RNA
SLAP: spacious *Listeria*-containing phagosome
SMS: sphingomyelin synthase
SPI: *Salmonella* pathogenicity island
STICK: substrates that interact with C kinases
TEM: transmission electron microscopy
TLR: Toll-like receptor
TNFα: tumor necrosis factor α
TTSS: type three secretion system
Ub: ubiquitin
UBA: ubiquitin-associated
UBZ: ubiquitin-binding zinc finger
ULK: unc-51-like kinase
UPS: ubiquitin-proteasome system
UVRAG: ultraviolet irradiation resistance-associated gene
VCC: *Vibrio cholera* cytolysin
VMP1: vacuole membrane protein 1
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Data in Figure 4-3A, B and C was generated with the assistance of Anton Namolovan. Quantification of data presented in Figure 4-7 was performed by Dr. Peter K. Kim.

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CHAPTER 1
INTRODUCTION

AUTOPHAGY

A) Forms of Autophagy

Autophagy is a degradative eukaryotic cell process that is evolutionarily conserved from yeast to mammals. This process is typically divided into three separate types that share some common components. The three types of autophagy include: macroautophagy, chaperone-mediated autophagy (CMA) and microautophagy. All three pathways terminate at the lysosome but the mechanism by which cargo is delivered differs between them. Macroautophagy is of significant interest to thesis and will therefore be the sole type of autophagy discussed.

Macroautophagy (hereafter referred to as autophagy), differs from the other two types of autophagy in that it involves the *de novo* formation of a membranous structure that grows to completely encircle its cargo within a double membrane structure. Initiation of this process requires the recruitment of core autophagy components to a site called the phagophore assembly site (PAS). Expansion of the PAS through the addition of membrane results in the formation of an enlarging phagophore that eventually completely encircles its cargo in a structure referred to as an autophagosome (AP) (Figure 1-1). APs mature through interaction with the endocytic pathway eventually fusing with lysosomes, resulting in the degradation of their contents. The macromolecules formed upon degradation of the AP cargo within the lysosome are transported back into the cytosol through membrane permeases providing substrates for recycling and reuse. Under normal conditions, autophagy is active at a basal level clearing damaged organelles and long-lived proteins. Upon stimulation by various stresses such as starvation, autophagy levels can be rapidly increased allowing for enhanced cell survival. Autophagy has been linked to multiple cell processes and will be described in detail in a separate section.
B) Components of the Mammalian Autophagy Pathway

The autophagy pathway involves over 30 distinct proteins ranging from protein kinases to phosphatidylinositol kinases and transmembrane proteins. The principal mammalian components of this pathway are typically divided into four distinct yet interconnected subgroups: the class III phosphatidylinositol 3-kinase (PI3K) human vacuolar protein sorting 34 (hVps34) complex, the unc-51-like kinase (ULK) complex, two ubiquitin-like conjugation systems and finally the transmembrane proteins mammalian Atg9 (mAtg9) and vacuole membrane protein 1 (VMP1) (Yang and Klionsky, 2010). All protein names discussed in this thesis refer to mammalian homologs unless otherwise described.

1. Class III Phosphatidylinositol 3-kinase hVps34 Complex

The core components of the PI3K complex are the PI3K hVps34, Beclin 1 (mammalian homolog of Atg6) and p150 (also known as Vps15) (Figure 1-2). The kinase activity of this complex results in the production of phosphatidylinositol 3-phosphate (PI3P) from phosphatidylinositol. This process is essential for autophagy, in fact, inhibition of this complex with pharmacological agents such as 3-methyladenine (3-MA), wortmannin and LY294002 is commonly used to inhibit autophagy (Klionsky et al., 2008). Mechanistically, the production of PI3P is thought to act to recruit PI3P-responsive autophagy components to the PAS such as WIPI or DFCP1, thereby promoting the induction of autophagy (Noda et al., 2010). The principal component of this complex is hVps34 which is a class III PI3K. hVps34 and its associated regulatory protein p150 interact with Beclin 1 although the function of this interaction is unknown (Funderburk et al., 2010).

The core kinase complex itself also interacts with other proteins in order to regulate autophagy. Interaction with Atg14L (also known as Barkor) is thought to function at the early stages of AP formation. Overexpression of Atg14L has been found to activate the kinase activity of this complex and as a result stimulate autophagy whereas knockdown has
Figure 1-1 – Mechanism of autophagosome formation.

Mammalian autophagy is thought to occur through the recruitment of autophagy components to the phagophore assembly site (PAS). This site grows through the addition of membrane to become a phagophore. The phagophore continues to expand and eventually encircles its cargo in a double membrane structure called an autophagosome. Autophagosomes mature through interaction with the endocytic pathway and eventually fuse with lysosomes. This fusion allows for the release of the inner autophagosomal membrane into this organelle. The degradative environment of this structure, called an autolysosome results in the breakdown of the membrane and autophagosome cargo. Vacuolar permeases allow for recycling and reuse of the resulting macromolecules. Figure adapted from (Yang and Klionsky, 2010).

Figure 1-2 – Class III phosphatidylinositol 3-kinase hVps34 complexes.

The core components of the Class III PI3K complex are hVps34, Beclin 1 and p150 (also known as Vps15). This core complex can interact with Atg14L or UVRAG. Interaction with these proteins is required for autophagy and is thought to function at the early stages of autophagosome formation. The core complex and UVRAG can also interact with Rubicon but this interaction negatively regulates autophagy. Figure adapted from (Funderburk et al., 2010).
been found to reduce PI3P production and inhibit autophagy (Sun et al., 2008; Zhong et al., 2009). The Beclin 1/hVps34/p150 complex also interacts with ultraviolet irradiation resistance-associated gene (UVRAG). Atg14L and UVRAG are not typically found on the same complex due to an overlap of Beclin 1 binding sites (Matsunaga et al., 2009). UVRAG has been found to play two different roles in autophagy regulation. One possible role involves interaction with the Beclin 1/hVps34 complex and is also thought to induce AP formation (Liang et al., 2006). UVRAG has also been found to interact with class C Vps/HOPS proteins on APs to stimulate the GTPase activity of Rab7, a process that aids in the maturation of APs through fusion with endosomes and lysosomes (Liang et al., 2008). Another protein that interacts with the Beclin 1/hVps34/p150 complex is RUN domain and cysteine-rich domain containing, Beclin 1 –interacting protein (Rubicon). Rubicon only binds to this complex in the presence of UVRAG suggesting an interaction with UVRAG (Matsunaga et al., 2009). The large complex of Beclin 1/hVps34/p150/UVRAG/Rubicon has been observed to associate with late endosomes/lysosomes and negatively regulate the maturation of APs (Matsunaga et al., 2009; Zhong et al., 2009).

2. ULK Complex

ULK is the mammalian homolog of the yeast serine/threonine kinase, Atg1. To date three isoforms of ULK have been identified: ULK1, ULK2 and ULK3. ULK1 and ULK2 have a strong similarity to Atg1 and have been better characterized than ULK3. ULK1 has been found to exist in complexes containing mammalian Atg13 (mAtg13), focal adhesion kinase family interacting protein of 200 kD (FIP200) and Atg101. ULK1, mAtg13, FIP200 and Atg101 are typically found within the cytosol but upon induction of autophagy localize to the PAS where they have all been found to be required for the formation of APs (Mizushima, 2010).

Depending upon nutrient status, this complex also interacts with mammalian target of rapamycin (mTOR) a central regulator of nutrient signalling (Hosokawa et al., 2009). mTOR is a serine/threonine kinase that is a negative regulator of autophagy (Ravikumar et al., 2004). Its interaction with the ULK complex results in various phosphorylation events
that regulate the entire complex. Under nutrient rich conditions, mTOR is active and
associates with the ULK complex, phosphorylating ULK1 and mAtg13. Phosphorylation of
ULK1 by mTOR inhibits ULK1’s own kinase activity. Under starvation conditions, mTOR is
inactive allowing for the activity of ULK1 which phosphorylates itself as well as mAtg13 and
FIP200 (Figure 1-3) (Mizushima, 2010). In addition to phosphorylating mAtg13 and FIP200
when active, ULK1 and ULK2 are also thought to phosphorylate other autophagy-related
proteins although their identity remains to be determined. This kinase activity of ULK1 and
ULK2 is thought to help recruit these proteins and allow for effective AP formation (Ganley
et al., 2009).

3. Ubiquitin-like Conjugation Systems

Efficient autophagy requires two inter-related conjugating systems that covalently
link Atg12 and microtubule-associated protein 1 light chain 3 (LC3) to distinct targets. These
pathways involve multiple proteins and are required for the expansion of growing APs. The
first conjugation system involves the linkage of Atg12 to another autophagy-related protein,
Atg5. This process requires the E1-like enzyme, Atg7 which activates Atg12. Atg12 is then
transferred to an E2-like enzyme, Atg10 and is ultimately conjugated to its target protein
Atg5 (Figure 1-4) (Geng and Klionsky, 2008). The E3-like enzyme that facilitates this final
step has yet to be identified. Additionally, Atg5 interacts noncovalently with another
protein, Atg16L (Matsushita et al., 2007). Atg16L oligomerizes with itself resulting in the
formation of a large tetramer complex of four Atg12-Atg5-Atg16L units (Kuma et al., 2002).
This complex typically localizes to the phagophore where it plays a role in the second
conjugation system.

The target of the second conjugation system, LC3 is initially translated in a pro-form,
processing to its active form requires the action of a protease (Atg4), which cleaves LC3 at
its C-terminus generating LC3-I . Both conjugation systems share an E1-like enzyme,
therefore Atg7 activates LC3 and transfers it to the E2-like enzyme, Atg3 (Yang and Klionsky,
2010). The complex of Atg12-Atg5-Atg16L acts as an E3-like enzyme, covalently linking LC3-I
Figure 1-3 – Serine/threonine ULK kinase complex.

The serine/threonine kinases ULK1 and ULK2 each form a complex with mAtg13 and FIP200. This complex is required for autophagy but its activity is regulated by the nutrient sensor kinase mTOR. Under normal nutrient conditions, mTOR phosphorylates ULK1/2 and mAtg13 resulting in inactivation of this complex. Under starvation conditions, mTOR is inactive and ULK1/2 is able to phosphorylate itself as well as mAtg13 and FIP200. The now active complex is recruited to the phagophore where it is thought to phosphorylate as yet unknown substrates thereby promoting autophagy. Figure adapted from (Yang and Klionsky, 2010).
**Figure 1-4 – Ubiquitin-like conjugation systems.**

The ubiquitin-like conjugation systems are required for expansion of the growing autophagosome. The first conjugation system involves the linking of Atg12 to Atg5. This involves the E1-like enzyme Atg7 and the E2-like enzyme Atg10. The E3-like enzyme required for ultimately conjugating Atg12 to Atg5 has yet to be identified. The complex of Atg12-Atg5 is able to interact noncovalently with Atg16L through binding via Atg5. This tripartite complex oligomerizes into a large tetrameric complex through homo-oligomerization of Atg16L. The second conjugation system involves the covalent linkage of LC3-I to phosphatidylethanolamine resulting in LC3-II. LC3-I is initially processed by the protease Atg4, which cleaves a C-terminal glycine. The now cleaved LC3-I is conjugated to phosphatidylethanolamine in a process requiring the E1-like enzyme Atg7, the E2-like enzyme Atg3. The complex of Atg12-Atg5-Atg16L acts as an E3-like scaffold, facilitating the final step of LC3 to phosphatidylethanolamine conjugation. Figure adapted and modified from (Maiuri et al., 2007)
to phosphatidylethanolamine (PE) to form the membrane-bound active LC3-II form (Figure 1-4) (Fujita et al., 2008). LC3-II associates with forming and complete APs both on the inner and outer membrane and is thought to be involved in the tethering and hemifusion of membrane (Nakatogawa et al., 2007). Due to its constant localization with APs, LC3 is commonly used as a specific marker of these structures by immunofluorescence analysis when using antibodies to LC3 or fluorescently tagged versions of this protein. Autophagy induction can also be analyzed biochemically by Western blot analysis. The two different forms of LC3: LC3-I and LC3-II run differently on a Western blot. Autophagy activation results in an induction of LC3-I to LC3-II conversion allowing for analysis of LC3-II levels correlating with autophagy induction (Klionsky et al., 2008).

4. Transmembrane Proteins and Associated Proteins

Autophagy involves the dramatic movement of membrane but among the over 30 autophagy-related proteins only two transmembrane proteins have been identified to date, mAtg9 and VMP1. Of the two, mAtg9 has been better characterized. mAtg9 is a ubiquitously expressed multi-spanning membrane protein with both N- and C-termini exposed to the cytosol. It typically localizes to the trans-Golgi network as well as to the cell periphery where it colocalizes with the endosomal protein markers Rab5, Rab7 and Rab9 (Webber and Tooze, 2010). mAtg9 localization changes under autophagy-inducing starvation conditions where it accumulates to a greater extent at the periphery and also colocalizes with LC3 (Young et al., 2006). This starvation-induced redistribution has been found to be ULK1 and hVps34-dependent (Young et al., 2006). In fact, antagonists of the PI3K hVps34 complex inhibit the redistribution of mAtg9 during starvation. Functionally, small interfering RNA (siRNA)-mediated knockdown of mAtg9 has been shown to decrease LC3-I to LC3-II conversion and impair autophagy but whether this is a direct or indirect outcome remains to be determined (Young et al., 2006). Another interesting functional role for mAtg9 involves the transport of membrane from the cell periphery to growing APs.
The second transmembrane protein required for autophagy is VMP1 (Ropolo et al., 2007). The subcellular location of VMP1 in mammalian cells has yet to be determined but it has been found to localize to the endoplasmic reticulum (ER) and be required for the maintenance of the ER and Golgi in Dictyostelium discoideum (Tooze, 2010). VMP1 has also been observed to colocalize with ULK1-positive puncta (Itakura and Mizushima, 2010). The specific role of VMP1 in autophagy has yet to be fully characterized but its expression is upregulated under starvation conditions. VMP1 has been observed to interact with Beclin 1 and is thought to function in AP formation, in fact, overexpression of this protein results in an increase in cellular AP numbers (Tooze, 2010).
AUTOPHAGY FUNCTION

Autophagy was initially found to play a role in the cellular response to stress and starvation and later in the clearance of long-lived proteins and damaged organelles. In addition to these cellular cargo, autophagy has also been found to mediate the degradation of endosomes/phagosomes, organelles such as mitochondria or peroxisomes, portions of the nucleus as well as the endoplasmic reticulum (Figure 1-5). The cellular roles autophagy plays can be broadly categorized into homeostasis, pro-survival and of importance for this thesis, immunity (Figure 1-6). In addition to organelle and protein turnover, autophagy's other homeostatic functions include programmed cell death and developmental remodelling (Boya et al., 2008; Mizushima and Levine, 2010). Autophagy's pro-survival function is typically in response to various environmental stressors such as starvation, growth factor withdrawal, oxidative stress and hypoxic conditions (Yang and Klionsky, 2010). This autophagic response allows for enhanced cell survival under these conditions. Autophagy also plays an integral role in innate and adaptive immune responses. This includes direct roles such as microbial degradation as well as indirect roles such as lysozyme secretion, antigen presentation and lymphocyte development (Deretic and Levine, 2009; Virgin and Levine, 2009). One target of this pathway that is of particular interest for this thesis is autophagy-mediated targeting of bacteria which will be discussed in detail in the next section.

While functioning in various cell processes, autophagy has also been linked to multiple human diseases including cancer, neurodegenerative diseases as well as myopathies. Autophagy can act as a double-edged sword whereby it can play a protective role against disease or in fact promote disease. For example, autophagy can play an anticancer role through the removal of damaged mitochondria and reducing chromosomal instability. On the other hand, autophagy can also benefit cancerous cells by providing resistance to therapeutic drugs and promoting cell survival in response to low nutrient levels (Mijaljica et al., 2010). At the same time, defective autophagy in and of itself has also
Figure 1-5 – Variety of cargos that can be targeted to the lysosome in an autophagy-dependent manner.

This model shows multiple types of autophagy including some yeast specific forms such as cytoplasm to vacuole targeting (CVT) and piecemeal microautophagy of the nucleus (PMN). The variety of mammalian autophagy targets range from endosomes to bacteria. Regardless of cargo and mechanism, all pathways terminate at the lysosome resulting in the degradation of cargo. Figure obtained from (Klionsky et al., 2007a)
Figure 1-6 – Cellular functions of autophagy.

Autophagy is understood to function in a variety of cellular responses and processes. The many roles of autophagy can be broadly divided into three categories. The first category falls under maintenance of cellular homeostasis, this includes general housekeeping functions such as the removal of damaged organelles or protein aggregates. The second category is as a pro-survival cellular response to stress. This can include responses to hypoxic or low nutrient conditions. The final category of autophagic functions involves cellular immunity. These immune related roles can be as part of the adaptive or innate immune system. Figure adapted from (Shahnazari and Brumell, 2011).
been associated with disease. A polymorphism in the Atg16L1 gene has been identified by genome wide association studies to be a risk factor for Crohn’s disease, a type of inflammatory bowel disease (Parkes et al., 2007). The role of autophagy in protection from and promotion of disease provides an attractive target for therapeutic intervention.

A) Autophagy and Bacterial Targeting

Direct antibacterial autophagy (also known as xenophagy) is appreciated to play a large role in the immune response to invading intracellular bacteria. An area of particular interest to us is the method by which our cells are able to recognize these foreign agents and specifically target them to this pathway. To date, numerous bacteria have been shown to be targeted by autophagy. Upon internalization, some pathogens can actively modify their vacuolar compartments in order to block maturation while others escape from their vacuolar compartments in order to replicate within the cytosol. The cell’s autophagic response can target pathogens at any step of this process, when they occupy intact or damaged vacuolar compartments or within the cytosol after escape has occurred (Figure 1-7).

APs are classically characterized as cytoplasmic cargo surrounded by an LC3-positive (LC3+) double membrane structure (Klionsky et al., 2008). It is difficult to accurately resolve this double-membrane structure by fluorescence light microscopy but using transmission electron microscopy (TEM) techniques, researchers have been able to identify APs. However, TEM micrographs of bacteria targeted by autophagy do not always resemble classical APs, in that they are not always double-membrane compartments. Contributions from bacterial toxins/effectors and disruption of endocytic trafficking can result in autophagy-associated multi-lamellar structures around bacteria. In some cases, single membrane APs can also be observed. In fact, latex bead phagosomes have been observed that are LC3+ but are comprised of only a single membrane (Sanjuan et al., 2007).

Importantly, all examples involve LC3 conjugation to membranes via components of the
Intracellular bacteria can occupy one of three niches. Intracellular bacteria such as *L. monocytogenes* can be present within the cytosol (1). Bacteria such as *M. tuberculosis* can occupy a vacuolar compartment (2). Finally, bacterial species such as *S. Typhimurium* can occupy a damaged vacuolar compartment (3). This damage can be a result of the actions of bacterial factors or a host response. Autophagy can target intracellular bacteria occupying any these different niches. The mechanism by which this occurs often requires different signals and signal mediators. Figure adapted from (Shahnazari and Brumell, 2011).
autophagy pathway, but the discrepancy in membrane structure complicates AP identification by TEM.

Bacteria can be targeted within intact vacuolar compartments. The first bacterial species found to be targeted within an intact membranous compartment was *Mycobacterium tuberculosis* (*M. tuberculosis*) (Gutierrez et al., 2004). Gutierrez et al. observed that upon physiological or pharmacological stimulation of autophagy, bacteria within a phagosomal compartment can be surrounded by a double-membrane structure (Figure 1-8a) and colocalize with LC3. The authors propose that induction of autophagy allows the host cell to overcome the block in phagosome maturation imposed by the bacteria and as a result negatively impact on mycobacterial survival (Gutierrez et al., 2004).

Bacteria can also be targeted within damaged compartments. Our lab has previously shown that autophagy of *Salmonella enterica* serovar Typhimurium (*S. Typhimurium*) requires bacterial protein synthesis and the *Salmonella* pathogenicity island (SPI)-1 type three secretion system (TTSS) in order to damage and escape from *Salmonella*-containing vacuoles (SCVs) (Birmingham et al., 2006). Autophagic targeting of these bacteria also requires both bacterial protein synthesis and a functional SPI-1 TTSS, suggesting that the bacteria are targeted in damaged SCVs. In support of this, LC3+ bacteria have been observed to colocalize with SCV markers including lysosomal-associated membrane protein 1 (LAMP1) (Birmingham et al., 2006). Zheng et al. observed that autophagy-targeted *S. Typhimurium* can be contained within multi-lamellar structures in wild type mouse embryonic fibroblasts (MEFs) while the majority of bacteria are within single membrane structures in autophagy-deficient, *atg5*−/− MEFs (Figure 1-8a) (Zheng et al., 2009). Autophagy targeting of this bacterial species was found to inhibit intracellular replication of *S. Typhimurium* (Birmingham et al., 2006).

Interestingly, two bacterial species that induce membrane damage to allow for their escape have been observed to be targeted both within phagosomes and also within the cytosol: *Listeria monocytogenes* (*L. monocytogenes*) and *Shigella flexneri* (*S. flexneri*). *L. monocytogenes* can escape into the cytosol, in a manner requiring the pore-forming toxin
A - Vacuolar targeting:

M. tuberculosis

1

B - Cytosolic targeting:

L. monocytogenes (ΔlacA)

1

F. tularensis

4

S. pyogenes

3

S. flexneri (ΔcsB)

2

S. flexneri

4

L. monocytogenes

1
Figure 1-8 – Transmission electron micrographs of bacterial targeting via autophagy.

(A – Vacuolar targeting) Bacteria targeted by autophagy within an intact or damaged vacuolar compartment. (A-1) *Mycobacterium tuberculosis* variant *bovis* BCG – Vacuolar *M. tuberculosis* within a double membrane autophagic structure (MAP – mycobacterial autophagosome) (Gutierrez et al., 2004). (A-2) *Salmonella enterica* serovar Typhimurium – S. Typhimurium within a multi-lamellar structure in WT MEFs (left panel). S. Typhimurium within a single membrane structure in *atg5*<sup>−/−</sup> MEFs (right panel) (Zheng et al., 2009). (A-3) *Listeria monocytogenes* – *L. monocytogenes* within a single membrane compartment termed a spacious *Listeria*-containing phagosome (Birmingham et al., 2008). (A-4) *Shigella flexneri* – Immuno TEM of *S. flexneri*, inset is a zoom-in of indicated area. Arrowhead corresponds with Galectin-3 while polyubiquitinated proteins indicated by an arrow (Dupont et al., 2009).

(B – Cytosolic targeting) Bacteria targeted by autophagy within the cytosol. (B-1) ∆*actA* *Listeria monocytogenes* – (a) cytosolic ∆*actA* *L. monocytogenes*. (b) crescent shaped double-membrane structure surrounding bacteria. (c) double membrane structure further elongating around bacteria. (d) cytosolic bacteria fully encompassed within a double membrane structure (Rich et al., 2003). (B-2) ∆*icsB* *Shigella flexneri* – ∆*icsB* *S. flexneri* completely surrounded within a multi-lamellar membranous structure (Ogawa et al., 2005). (B-3) *Streptococcus pyogenes* – Immuno TEM of GFP-LC3<sup>+</sup> Group A *Streptococcus*-containing autophagosome-like vacuoles (GcAVs) (Yamaguchi et al., 2009). (B-4) *Francisella tularensis* – Multiple *F. tularensis* within a double membrane structure (Checroun et al., 2006). Figure adapted from (Shahnazari and Brumell, 2011).
listeriolysin O (LLO) and two phospholipase C enzymes. Once within the cytosol, these bacteria mediate actin polymerization and movement through the bacterial protein ActA (Dussurget, 2008). Birmingham et al. observed that a sub-population of these bacteria do not produce enough LLO to allow for efficient escape. They observed that this subpopulation is targeted by the autophagy pathway resulting in the formation of LC3+ single-membrane spacious Listeria-containing phagosomes (SLAPs) (Figure 1-8a) (Birmingham et al., 2008). These compartments are suggested to be the causative agent for persistent Listeria infection as the bacteria are still able to replicate in vivo within these large compartments.

The first example of autophagic-targeting of bacteria within the cytosol was that of the ΔactA mutant of L. monocytogenes. Rich et al. observed that in the presence of chloramphenicol (a bacteriostatic antibiotic), cytosolic ΔactA L. monocytogenes are surrounded by a double-membrane autophagosomal structure (Rich et al., 2003). Autophagy is a dynamic process, but EM micrographs only provide a snapshot of events. Interestingly, Rich et al. were able to show what appears to be initiating, intermediate and complete autophagic structures (with classical double-membrane morphology) growing to surround and encompass the cytosolic bacteria (Figure 1-8b).

S. flexneri also induces membrane damage to allow for bacterial escape into the cytosol. At the same time, this bacteria has developed a strategy to avoid bacterial targeting within the cytosol. In a seminal paper, Ogawa et al. observed that efficient autophagy of S. flexneri occurs through the interaction of the autophagy protein Atg5 with the surface bacterial protein VirG. The secreted bacterial effector IcsB competitively binds to VirG, thereby blocking autophagic targeting (Ogawa et al., 2005). ΔicsB S. flexneri were observed to have decreased bacterial replication compared to wild type and to associate with multi-lamellar structures (Figure 1-8b) (Ogawa et al., 2005). These bacteria can also be targeted by autophagy prior to escape. Dupont et al. observed that phagosome membrane remnants (as marked by Galectin-3 staining) during escape can be targeted by autophagy (Figure 1-8a) (Dupont et al., 2009).
Streptococcus pyogenes (S. pyogenes) and Francisella tularensis (F. tularensis) have also been observed to be targeted by autophagy within the cytosol but with very different outcomes. S. pyogenes escapes phagosomal compartments through secretion of the pore-forming toxin streptolysin O but can re-enter the endocytic pathway through containment and subsequent bacterial killing within Group A Streptococcus-containing autophagosome-like vacuoles (GcAVs) that mature through fusion with lysosomes (Nakagawa et al., 2004). These GcAVs are LC3+ and contain multiple bacteria (Figure 1-8b) (Nakagawa et al., 2004; Yamaguchi et al., 2009).

F. tularensis escape their phagosomes and are able to replicate within the cytosol. Checroun et al. observed that these bacteria are able to re-enter the endocytic pathway within LAMP1+ and LC3+ structures called Francisella-containing vacuoles (FCVs) (Checroun et al., 2006). FCVs seem to be comprised of multiple membranes and contain multiple bacteria (Figure 1-8b). While autophagy was found to be required for the generation of these structures, the bacteria were observed to be intact even following lysosomal fusion (Checroun et al., 2006).

The mechanisms by which bacteria are targeted appear to be diverse. It can depend on the microbe targeted and can include numerous mechanisms. This is not surprising considering the fact that multiple sources of membrane have been proposed for the formation of APs (Tooze and Yoshimori, 2010). I will next describe the known signals and signaling mediators that have been implicated in the targeting of bacteria by autophagy.

1. Toll-like and NOD-like Receptors

Pathogen-associated molecular patterns (PAMPs) such as lipopolysaccharides (LPS) are detected by components of the innate immune system and include pattern recognition receptors (PRRs) such as transmembrane Toll-like receptors (TLRs) and cytosolic nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs) (Bortoluci and Medzhitov, 2010). Multiple studies have shown that TLRs can activate autophagy. Xu et al. also observed that TLR4 stimulation with LPS resulted in autphagic induction and increased
colocalization of \textit{M. tuberculosis} with APs (Xu et al., 2007). Delgado \textit{et al.} similarly observed that TLR7 activation with ssRNA resulted in enhanced \textit{M. tuberculosis} clearance (Delgado \textit{et al.}, 2008). While these studies showed an induction of autophagy they did not show a direct connection between PRRs and autophagy.

One study that indicated a potential targeting role via PRRs was by Sanjuan \textit{et al.} (Sanjuan \textit{et al.}, 2007). They observed that stimulation of TLR2 with zymosan during phagocytosis induced the recruitment of LC3 to the phagosome in an Atg5 and Atg7-dependent manner (Sanjuan \textit{et al.}, 2007). Interestingly, they showed that LC3$^+$ phagosomes had a single membrane, unlike classical APs (Sanjuan \textit{et al.}, 2007). The mechanism by which PRRs are able to promote antibacterial autophagy is currently unclear, though production of reactive oxygen species (ROS) is required and will be discussed in the next section. Another direct connection between PRRs and autophagy has been observed with NLRs. Travassos \textit{et al.} found that the peptidoglycan detectors Nod1 and Nod2 directly localized to sites of \textit{S. flexneri} entry and promoted the recruitment of the autophagy component Atg16L1 to these sites via a direct interaction (Travassos \textit{et al.}, 2010). Autophagy of \textit{S. flexneri} was impaired in the presence of Nod2 mutants defective in peptidoglycan sensing (NOD2 L1007insC, Nod2fs), a polymorphism prevalent in Crohn’s disease (Travassos \textit{et al.}, 2010). The fact that Nod1 and Nod2 are cytosolic and detect bacterial peptidoglycan could indicate that there is exposure of the bacteria to the cytosol. Whether these sensors function at damaged phagosomes remains to be determined.

2. Reactive Oxygen Species

There are many sources of ROS in mammalian cells, including those produced by the mitochondria and through the action of NADPH oxidases (NOXs) (Valko \textit{et al.}, 2007). ROS include superoxide, hydroxyl radicals and hydrogen peroxide. Mitochondrial ROS, have been previously found to play a role in starvation-induced autophagy (Scherz-Shouval \textit{et al.}, 2007).
Activation of TLRs and Fcγ receptor (FcγR) results in the generation of NOX-generated ROS at the forming phagosome (Quinn and Gauss, 2004). In immune cells, NOX2 NADPH oxidases are a major source of ROS. Huang et al., found that NOX-generated ROS plays a positive role in the autophagy of S. Typhimurium in human intestinal epithelial cells (Huang et al., 2009). They initially observed that stimulation of TLR4, TLR2 and FcγR in RAW macrophages with LPS, zymosan and human IgG coated latex beads respectively, resulted in LC3 recruitment to the phagosome. Further work with antioxidants and WT and NOX2−/− primary bone marrow-derived neutrophils revealed a critical role for ROS in the recruitment of LC3 to the phagosome in response to PRR activation (Huang et al., 2009). siRNA-mediated knockdown of a key NOX component, p22 resulted in a significant decrease in autophagy of S. Typhimurium in human intestinal epithelial cells (Huang et al., 2009). The mechanism by which a localized ROS signal is mediated and how it is able to specifically recruit LC3 to the phagosome remains to be determined. Interestingly, Huang et al. found that the Atg5-Atg12 conjugate was present on the phagosome (Huang et al., 2009). This suggests that LC3 is conjugated directly to the phagosome membrane in response to a ROS-generated signal at this compartment. How ROS are able to induce the recruitment of these and potentially other autophagy components is unknown.

3. Ubiquitin

One of the most well characterized signals for autophagic targeting of bacteria, or other cargo for that matter, is conjugation to ubiquitin (Ub). Ub is a small (76 amino acid) protein originally characterized as a protein label that targets tagged proteins for degradation by the 26s proteasome via a system termed the Ub-proteasome system (UPS) (Shabek and Ciechanover, 2010). Ub can be present as a monomer or as a polymer chain with polymers formed via linkage between lysine 48 (K48) Ub residues being associated with targeting to the proteasome for degradation (Pickart, 1997). The conjugation of a Ub moiety to a substrate requires the activity of three enzymes: an E1 activating enzyme, an E2 conjugating enzyme and finally an E3 Ub ligase (Shabek and Ciechanover, 2010). While Ub
has been found to act as a signal, initiating autophagy of tagged substrates, this process requires adaptors capable of connecting the Ub signal to the autophagy pathway. To date six such adaptors have been identified: p62/SQSTM1, nuclear dot protein 52 (NDP52), neighbor of BRCA1 gene 1 (NBR1), HDAC6, ALfy and BAG3 (Kraft et al., 2010). Of these, only three Ub/autophagy adaptors directly mediate an interaction between ubiquitinated-targets and LC3: p62/SQSTM1, NDP52 and NBR1. Direct Ub binding is achieved through Ub-associated (UBA) or Ub-binding zinc-finger (UBZ) domains. LC3/adaptor interaction is mediated by LC3-interaction regions (LIRs) which may aid in the recruitment and organization of LC3+ membrane at the target.

The first link between ubiquitination and the autophagy pathway involved the selective degradation of ubiquitinated protein aggregates. Initially, this was shown to require the action of p62/SQSTM1 (Bjorkoy et al., 2005), but more recently NBR1 has also been found to play a role in the selective degradation of ubiquitinated proteins (Kirkin et al., 2009). The first bacterium identified as being targeted by autophagy in a Ub-dependent manner was S. Typhimurium. It was initially shown that 50% of autophagy-targeted (LC3+) S. Typhimurium also colocalized with mono- and polyubiquitinated proteins (Birmingham et al., 2006). Efficient autophagy of ubiquitinated S. Typhimurium has been recently shown to require the action of two different Ub/LC3 adaptors: p62/SQSTM1 and NDP52 (Thurston et al., 2009; Zheng et al., 2009). The reason why two different adaptors are required for efficient autophagy of the bacteria is not clear. It is of note that ubiquitinated S. Typhimurium still colocalizes with membrane markers including LAMP1, indicating that phagosomal membrane is still present and the bacteria may occupy a damaged membranous compartment (Birmingham et al., 2006).

In contrast to S. Typhimurium, Ub-mediated targeting of L. monocytogenes to the autophagy pathway occurs within the cytosol. ΔactA L. monocytogenes was originally observed to be targeted by the autophagy pathway within the cytosol of infected cells when in the presence of the bacterial protein synthesis inhibitor chloramphenicol (Rich et al., 2003). This pathway has been further elucidated to show a role for ActA in protection of L. monocytogenes from autophagic targeting (Yoshikawa et al., 2009). Yoshikawa et al.
observed that ActA functions to recruit host actin-polymerizing complexes to the bacteria thereby protecting it from targeting. Cytosolic bacteria expressing defective ActA were found to be targeted by autophagy in a manner requiring protein ubiquitination and p62/SQSTM1 recruitment (Yoshikawa et al., 2009).

Questions that remain to be answered in terms of Ub-mediated bacterial autophagy targeting include the identity of the ubiquitinated protein(s) associating with S. Typhimurium and ActA-defective L. monocytogenes. This could include host or bacterial proteins, or a combination of both. Another intriguing question is the identity of the species of Ub to which these adaptors bind; Ub can be present as a monomer or as polymer chains (K27, K48 or K63 linkages). Additionally, to date hundreds of E3 ligases have been identified in mammalian cells as well as bacterial pathogens, the identity of the specific ligase(s) required for this process also remain to be determined (Collins and Brown, 2010). The possibility also exists that other intracellular bacteria can be targeted in a Ub-mediated fashion, either within damaged compartments such as S. Typhimurium or within the cytosol like L. monocytogenes.

Invading/intracellular bacteria can be targeted by autophagy within the cytosol, intact or even damaged membrane-bound compartments. This provides the cell with multiple avenues for the specific targeting of bacteria to the autophagy pathway. The outcome of this targeting can be beneficial to the host or pathogen, depending on both environmental and genetic factors leading up to infection. While autophagy is clearly an innate immune defence under many conditions, some pathogens can evade or even exploit this host mechanism. Indeed, the establishment of persistent bacterial infections may involve autophagy (Birmingham et al., 2008). While I have described known mechanisms that can target bacteria in each of these niches, I anticipate other factors will be found that contribute to these targeting events. Whether these factors contribute to autophagy regulation via the same or independent pathways is currently unclear, and will require further study. A number of bacterial virulence factors have been identified to impair autophagy targeting and/or autophagosome maturation. The mechanisms by which these factors function, in most cases, remains unknown and will also be the subject of intensive
study. It is conceivable that many more bacterial virulence factors will be found to block/modulate autophagy, since it represents a key aspect of host immunity.
SECOND MESSENGERS

Second messengers are molecules that transduce extracellular signals that are received by cell surface receptors. Activation of these receptors result in the generation of various second messengers which act to relay and enhance this signal. Two well characterized second messengers are diacylglycerol and cyclic adenosine monophosphate.

A) Diacylglycerol

Diacylglycerol (DAG) is a lipid composed of a glycerol backbone and two covalently linked fatty acid chains. It is a unique molecule in that it plays multiple cellular roles including: an intermediate in lipid biosynthesis, a membrane-associated signalling molecule and finally as a structural component of membranes. The multiple roles of DAG necessitate a requirement for tight control of both its generation and signalling function.

1. Diacylglycerol Generation and Lipid Metabolism

Because of its structural simplicity, DAG can act as the building block of many different structural and signalling lipids. Modification of DAG through the addition of various headgroups can lead to the production of the energy source triacylglycerol as well as phosphatidylinositol (PI), phosphatidylserine (PS), phosphatidylethanolamine (PE) and phosphatidylcholine (PC).

DAG itself lies at the centre of a biosynthetic pathway whereby it can be generated through the enzyme-mediated modification of various lipids. DAG can be generated through the dephosphorylation of phosphatidic acid (PA) by phosphatidic acid phosphatase (PAP). DAG can also be generated by phospholipase C (PLC) which cleaves phosphatidylinositol 3,4-bisphosphate releasing an inositol 3,4,5-trisphosphate group. A third pathway involves sphingomyelin synthase (SMS) which converts phosphatidylcholine to sphingomyelin releasing DAG and ceramide (Figure 1-9) (Carrasco and Merida, 2007).
**Figure 1-9 – Biosynthetic pathways for the production of diacylglycerol.**

Diacylglycerol (DAG) production can occur through one of three biosynthetic pathways. This includes the conversion of phosphatidylcholine (PC) to phosphatidic acid (PA) by phospholipase D (PLD) and its subsequent dephosphorylation to DAG by phosphatidic acid phosphatase (PAP). DAG can also be generated by phospholipase C (PLC) acting on phosphatidylinositol 4,5-bisphosphate (PI4,5P2). Finally, DAG can be generated from PC and ceramide by sphingomyelin synthase (SMS) releasing sphingomyelin (SM) and DAG.
Since DAG plays a signalling role, cells maintain tight control of intracellular DAG levels. DAG can be modified by diacylglycerol kinases (DGKs) to produce PA. DAG can also be converted to triacylglycerol in a reaction catalyzed by diacylglycerol acyltransferases. As previously mentioned, DAG can also be processed to form PC and PE, this reaction is catalyzed by choline/ethanolamine phosphotransferase and choline phosphotransferase. The importance of DAG in lipid metabolism is evident in the variety of forms that it can be modified to.

2. Diacylglycerol-mediated Signalling and Effectors

As a membrane-associated signalling lipid, DAG functions to recruit responsive proteins to the membrane surface. Proteins able to bind DAG contain at least one C1 domain. C1 domains are 50 amino acids in length consisting of a HX$_{11-12}$CX$_2$CX$_{12-14}$CX$_2$CX$_{6-7}$C motif (Yang and Kazanietz, 2003). C1 domains themselves can be typical or atypical, with typical C1 domains being DAG-responsive (Colon-Gonzalez and Kazanietz, 2006). There are six protein families containing at least one typical C1 domain. Classical protein kinase C (PKC) members include $\alpha$, $\beta$I, $\beta$II and $\gamma$ isoforms while novel PKC members include $\delta$, $\epsilon$, $\eta$ and $\theta$ isoforms (Carrasco and Merida, 2007). Both classical and novel PKC isoforms contain two typical C1 domains. PKC isoforms are of significant interest for this thesis and will be described in detail in a separate section. Another family of serine/threonine protein kinases that contain two typical C1 domains are members of the protein kinase D (PKD) family that include PKD1, 2 and 3 isoforms. DGK isoforms $\beta$ and $\gamma$ also contain two typical C1 domains (Figure 1-10). Four other DAG-responsive protein families exist that only contain single typical C1 domains. This includes multiple isoforms of the Rac GTPase-activating protein chimaerin family, the scaffold protein mammalian unc13, and the Ras guanine nucleotide exchange factor RasGRP (Colon-Gonzalez and Kazanietz, 2006) (Figure 1-10). Due to the variety of DAG effectors, this lipid plays a diverse signalling role. DAG-induced cellular responses can range from proliferation and survival to vesicle trafficking, immune cell signalling, cell motility and exocytosis (Toker, 2005).
Figure 1-10 – Schematic of diacylglycerol-responsive protein families.

DAG-responsive proteins all contain at least one C1 domain. Conventional protein kinase C (cPKC), novel PKC (nPKC), protein kinase D (PKD) and diacylglycerol kinase (DGK) isoforms contain two DAG-responsive C1 domains. RasGRP and chimaerin families as well as mammalian unc13 (Munc13) contain single DAG-responsive C1 domains. The other depicted protein domains include: C2 (phospholipid binding), pleckstrin homology (PH), EF hand (EF), guanine nucleotide exchange (GEF), Ras exchanger motif (REM), GTPase-activating protein (GAP). Figure adapted from (Toker, 2005)
3. Diacylglycerol as a Structural Component

In addition to its metabolic and signalling roles, DAG also plays a structural role in cellular membranes. Depending on headgroup and tail size, lipids typically take one of three shapes inducing a specific type of membrane curvature. Lipids with headgroups and acyl tails that are similar in size, act as cylinders and do not impart any curvature to membranes. Lipids with large headgroups but small acyl tails act as inverted cones and induce positive curvature while lipids such as DAG that have small headgroups and large acyl tails act as cones and induce negative curvature in membranes. Negative curvature in membranes is beneficial because it allows for the formation of areas of increased flexibility and instability facilitating membrane fusion and fission (Shemesh et al., 2003).

B) Cyclic Adenosine Monophosphate

Cyclic adenosine monophosphate (cAMP) is the prototypical second messenger that acts as a conserved regulator of cell function in a wide range of organisms ranging from plants to mammals (Hofer and Lefkimmiatis, 2007). cAMP signalling can modulate multiple cellular processes including: memory, metabolism, gene regulation and immune function (Beavo and Brunton, 2002).

1. cAMP Generation

cAMP is typically generated upon stimulation of G protein-coupled receptors (GPCRs) by the extracellular binding of a first messenger such as neurotransmitters, hormones, chemokines or pharmacological agents (Serezani et al., 2008). Activated GPCRs catalyze the activation of various associated G protein α subunits (Gα) through exchange of guanosine diphosphate (GDP) for guanosine triphosphate (GTP) resulting in the dissociation of this subunit (Landry et al., 2006). Depending on the identity of the dissociating subunit, one of two outcomes can occur. Activated Gαs binds to and activates adenylate cyclase (AC)
which catalyzes the generation of cAMP from adenosine triphosphate (ATP) (Figure 1-11). At the same time, activation of Gαi-linked GPCRs and subsequent dissociation of this specific subunit results in down-regulation of AC and a decrease in cAMP levels (Murray, 2008). There exist at least ten known isoforms of AC that are expressed at different levels in various tissues (Sunahara and Taussig, 2002).

Similar to DAG, intracellular cAMP levels are strictly controlled. cAMP has the ability to diffuse throughout the cell and indiscriminately activate its effectors necessitating a mechanism to control subcellular cAMP concentration (Zaccolo et al., 2006). In addition to control of cAMP generation via AC, this second messenger can also be degraded through the actions of phosphodiesterases (PDEs) which hydrolyze the cAMP phosphodiester bond converting it to AMP (Figure 1-11). One of the principal mechanisms by which compartmentalization of cAMP signalling occurs is through control of subcellular PDE localization. The N-terminus of PDEs determine their subcellular localization which can be within the cytosol, at the plasma membrane (PM) or on organelles (Adderley et al., 2010).

2. cAMP Signalling and Downstream Effectors

CAMP can play a role in the modulation of many cellular processes. Its actions are mediated by three principal effectors: exchange protein directly activated by cAMP (Epac), cyclic nucleotide-gated ion channels (CNGCs) and the serine/threonine protein kinase A (PKA) (Murray, 2008). Through these effectors, cAMP is able to negatively regulate multiple processes including immune function (Serezani et al., 2008). Most immunosuppressive phenotypes associated with cAMP elevation have been attributed to the actions of either Epac (Aronoff et al., 2005; Xu et al., 2008) or PKA (Aronoff et al., 2006; Bengis-Garber and Gruener, 1996; Bryn et al., 2006; Serezani et al., 2007) (Figure 1-11).

Epac is a guanine nucleotide exchange factor for the small G proteins Rap1 and Rap2 (Gloerich and Bos, 2010). It has been linked to inhibition of FcγR-mediated phagocytosis in alveolar macrophages (Aronoff et al., 2005). In liver macrophages, Epac has been found to inhibit pathogen-associated ROS generation (Usynin et al., 2007). Epac can also play a role
Figure 1-11 – Cyclic adenosine monophosphate generation and signalling.

Cyclic adenosine monophosphate (cAMP) is typically generated in response to activation of G-protein coupled receptors (GPCRs). Activation of Gαs-linked GPCRs results in the activation and release of Gαs which activates adenylate cyclase (AC). AC catalyzes the conversion of adenosine triphosphate (ATP) to cAMP. On the other hand, activation of Gαi-linked GPCRs can lead to AC inhibition. The majority of cAMP signalling occurs through exchange protein directly activated by cAMP (Epac) and protein kinase A (PKA). cAMP can be degraded through the action of phosphodiesterases (PDEs) which convert cAMP to AMP. Certain bacterial factors are able to increase cellular cAMP through modulation of host machinery. Cholera toxin from Vibrio cholera is an ADP-ribosyltransferase capable of activating Gαs subunits. On the other hand, Pertussis toxin from Bordetella pertussis is also an ADP-ribosyltransferase that inactivates Gαi subunits. Figure adapted from (Serezani et al., 2008)
in regulation of inflammation by negatively regulating the production of pro-inflammatory chemokines by leukocytes in response to pathogens (Gloerich and Bos, 2010). Interestingly, Epac has also been found to play a pro-inflammatory role by enhancing integrin-mediated leukocyte adhesion and migration (Bos, 2005). In addition to its role in immune function, Epac has been found to be involved in cardiac function, insulin secretion as well as neuronal, renal and vascular function (Gloerich and Bos, 2010). Similar to Epac, PKA also plays a large role in mediating the transduction of cAMP signalling. Due to the significance of this protein kinase to this thesis, I will describe its function in detail in a separate section.

C) Bacterial Elevation of Cyclic Adenosine Monophosphate Levels in Host Cells

Numerous bacterial pathogens have evolved mechanisms that allow them to exploit the regulatory functions of cAMP in order to suppress immune responses (Baldari et al., 2006). Bacterial modulation of intracellular cAMP levels can occur via two different mechanisms: direct production of cAMP or modulation of host cAMP-regulating machinery.

1. Direct cAMP Production

A bacterial species that contains a virulence factor that can directly synthesize cAMP is *Bacillus anthracis* (*B. anthracis*). This Gram-positive, aerobic, spore-forming pathogen is the causative agent of anthrax. *B. anthracis* virulence is mediated by a three-component exotoxin system. These toxins are comprised of two ‘AB’ toxin pairs that share a common B component, protective antigen (PrA) which is a cell-surface binding moiety that allows for the internalization of the associating toxin. The combination of PrA and lethal factor (LF) is referred to as lethal toxin (Ltx) while the combination of PrA and edema factor (EF) is known as edema toxin (Edtx) (Leppla, 1982).

Typically, PrA binds to cell surface receptors such as Anthrax toxin receptor 1 and is cleaved by cell surface proteases such as furin resulting in the release of a 20 kDa fragment. The remaining 63 kDa PrA fragment oligomerizes forming a pore-shaped heptmer (Elliott et
al., 2000). LF or EF compete for three binding sites exposed upon proteolysis and oligomerization of PrA (Mogridge et al., 2002). The resulting complex of PrA and LF and/or EF is internalized via clathrin-mediated endocytosis, with acidification of the resulting endosome resulting in a conformational change in the structure of the heptamer allowing for translocation of the associated LF or EF molecules into the host cytosol (Young and Collier, 2007).

Once within the cytosol, LF or EF are able to perform their toxic function. LF is a metalloprotease that can cleave the N-terminal tail of several isoforms of mitogen-activated protein kinase kinases thereby negatively affecting cell signalling (Tonello and Montecucco, 2009). Of significant interest for this thesis is the second toxin, EF which is a calcium/calmodulin-dependent AC that can directly convert ATP to cAMP thereby raising intracellular cAMP levels (Ahuja et al., 2004). The immunosuppressive function of cAMP has been described earlier, similarly, EF-derived cAMP has been found to have a similar effect. In monocytes, EF-induced cAMP has been found to inhibit synthesis of LPS-induced tumor necrosis factor-α production (Hoover et al., 1994). In neutrophils, elevated B. anthracis-induced cAMP levels has been observed to inhibit LPS-induced neutrophil priming as well as phagocytosis (Ahuja et al., 2004). In addition to B. anthracis, Bordetella pertussis, Mycobacterium tuberculosis and Pseudomonas aeruginosa also produce AC toxins (Agarwal et al., 2009; Hewlett et al., 1976; Yahr et al., 1998). By negatively affecting various immune related cell functions via cAMP elevation, these bacterial ACs may allow for increased bacterial survival and replication.

2. Activation of Host cAMP-producing Machinery

In addition to directly synthesizing cAMP, some bacterial species are able to activate host cell AC machinery thereby indirectly increasing intracellular cAMP levels (Figure 1-11). One such bacterial species is the Gram-negative gastrointestinal pathogen, Vibrio cholera (V. cholera) which is the causative agent of the acute diarrheal disease, cholera. V. cholera virulence is mediated by a heterodimeric ‘AB’ toxin, cholera toxin (Ctx). Ctx is comprised of
a single A subunit and five B subunits. The B subunits each contain a single binding site that allows the complex to interact with the apical cell surface of enterocytes via binding to the monosialoganglioside GM₁ (Chinnapen et al., 2007). This interaction and subsequent internalization and retrograde transport via the Golgi to the ER and the cytosol results in dissociation of the catalytic A subunit. The Ctx A subunit is an ADP-ribosyltransferase that catalyzes the ADP-ribosylation of Gαs resulting in its constitutive activation (De Haan and Hirst, 2004). Activated Gαs binds to host ACs resulting in constitutive production of cAMP.

While Ctx primarily targets intestinal epithelial cells, it has also been found to affect leukocytes. Ctx has been shown to reduce FcγR-mediated phagocytosis in polymorphonuclear leukocytes, inhibit bacterial killing and interleukin-12 production in monocytes and inhibit polymorphonuclear neutrophil migration (Bergman et al., 1978; Braun et al., 1999; Niemialtowski et al., 1993; O'Dorisio et al., 1979). In addition to V. cholera, other pathogens including enterotoxigenic Escherichia coli and Bordetella pertussis also produce ADP-ribosyltransferases capable of activating host ACs by activating Gαs or inhibiting Gαi respectively (Figure 1-11) (Carbonetti et al., 2005; Fleckenstein et al., 2010a).
PKC family members are a typically membrane-localized serine/threonine protein kinase group that comprise approximately 2% of the human kinome (Rosse et al., 2010). Classical, novel and atypical PKC isoforms all share a conserved C-terminal kinase domain that is connected to a variable N-terminal regulatory domain. This isoform-specific variable regulatory region allows for controlled activation of PKC providing for spatial control of function. PKC is normally present in an auto-inhibited state due to an interaction between a pseudosubstrate sequence in the regulatory region and the substrate binding pocket of the kinase domain (Pears et al., 1990). Second messenger or allosteric effector binding allows for displacement of the pseudosubstrate region resulting in activation of PKC. As described previously, classical and novel PKC family members contain two C1 domains. Classical PKC isoforms can be activated by DAG, phospholipids and Ca\(^{2+}\) ions. Novel PKC isoforms are similarly activated by DAG and phospholipids but not Ca\(^{2+}\). Finally, atypical PKCs including ζ and ι isoforms contain a single C1 domain which is not DAG-responsive, these isoforms are allosterically activated via an interaction with a complex containing partitioning defective 6 and cdc42 (Suzuki et al., 2001).

1. Protein Kinase C Function

PKC isoforms are typically ubiquitous and found in multiple tissues (Liu and Heckman, 1998). Since they are membrane associated they can be found on multiple membranous compartments including the plasma membrane, endosomes, the Golgi as well as the nucleus (Parker and Murray-Rust, 2004). Due to their kinase activity, PKC isoforms have been implicated in a variety of signalling roles in an isoform, tissue and stimulation-specific manner. PKC specificity and function is mediated by a variety of interacting proteins including receptors for activated C kinases (RACKs) and substrates that interact with C
kinases (STICKs) (Poole et al., 2004). The roles of PKC include control of cell proliferation, adhesion, migration, differentiation, survival, apoptosis, transformation as well as regulation of cell-cell contact and immune responses (Rosse et al., 2010; Yang and Kazanietz, 2003).

Due to this variety of function, the pool of PKC substrates is quite extensive. For example, PKC isoforms actively regulate the Raf- mitogen-activated protein kinase kinase (MEK) – extracellular signal-regulated kinase (ERK) pathway. PKCα can directly phosphorylate Raf-1, activating this pathway thereby promoting proliferation (Kolch et al., 1993). PKCs can also activate nuclear factor -κB (NF-κB)-mediated transcription through activation of inhibitor of κB kinases (IKKs) (Saijo et al., 2002). Other PKC substrates include vinculin, nucleolin, ezrin, adducin and dynamin (Parker and Murray-Rust, 2004).

2. Protein Kinase C and Autophagy

In addition to the aforementioned roles, recent work has identified a regulatory function for PKC in autophagy. Sakaki et al. found that ER stress-induced autophagy required the activation of the novel PKC, PKCθ (Sakaki et al., 2008). They initially observed that treatment of cells with the ER stressors thapsigargin (inhibitor of sarco/endoplasmic reticulum Ca^{2+} ATPase) or tunicamycin (disrupts glycosylation of newly-synthesized protein) resulted in the induction of autophagy. They found that this induction of autophagy was PKCθ-dependent and that PKCθ was activated under these conditions. They also found that PKCθ localized to LC3+ structures within the cell. The role for this isoform was specific to ER-stress induced autophagy and not autophagy induced in response to starvation. The mechanism by which PKCθ is activated in response to these stresses, the identity of its substrates and how it is able to localize to LC3+ structures has yet to be determined.

Another PKC isoform implicated in autophagy is PKCδ. This isoform was found to be involved in hypoxic stress-induced autophagy (Chen et al., 2008). Chen et al. observed that acute hypoxic stress resulted in a PKCδ-dependent induction of autophagy. The precise
mechanism by which PKCδ is able to activate autophagy and the process by which it itself is activated remains to be fully characterized. PKC has also been found to be involved in the autophagic response during sulfaphenozole (an inhibitor of cytochrome P4502C9)-mediated cardioprotection against ischemia-reperfusion injury (Huang et al., 2010). The role and mechanism of function for other PKC isoforms in other types of autophagy remains to be determined.

B) Protein Kinase A

PKA was one of the earliest protein kinases to be discovered (Walsh et al., 1968). While similar to PKC in that it is a serine/threonine protein kinase, PKA differs from the majority of other protein kinases as it is a heterotetrameric holoenzyme composed of two catalytic and two regulatory subunits. The catalytic subunits are initially phosphorylated within the activation loop by phosphoinositide-dependent protein kinase 1 (PDK1) at T197, a process which is necessary for the maturation of this protein (Cheng et al., 1998). The activity of the now active catalytic subunit is subsequently regulated through an interaction with the regulatory subunit. The binding of two cAMP molecules to each regulatory subunit results in a conformational change and release of the catalytic subunit (Figure 1-12) (Cheng et al., 2008). There exist two distinct PKA isoforms: PKA-I and PKA-II that differ in their regulatory subunits. PKA-I interacts with RI regulatory subunits whereas PKA-II interacts with RII regulatory subunits (Naviglio et al., 2009). Both RI and RII subunits bind cAMP but differ in their N-terminal regions. In addition to the regulatory subunits, PKA is also regulated by A-kinase anchoring proteins (AKAPs). AKAPs play a role in the differential localization of PKA-I and PKA-II within the cell. AKAPs interact with the N-terminal region of the regulatory subunits tethering the holoenzyme to various subcellular locations (Huang et al., 1997).
Protein kinase A (PKA) is a heterotetrameric holoenzyme consisting of two regulatory (R) subunits and two catalytic (C) subunits. Activation of G-protein coupled receptors (GPCR) and adenylate cyclase (AC) results in an increase in intracellular cAMP levels. The binding of two cAMP molecules to each R subunit of PKA results in the dissociation of the C subunits. The free C subunits are subsequently able to phosphorylate various substrates. Figure adapted from (Gerits et al., 2008)
1. Protein Kinase A Function

PKA is one of the principal mediators of cAMP signalling. Free catalytic subunits are able to phosphorylate a wide variety of substrates both within the nucleus as well as the cytoplasm. PKA has been shown to regulate intracellular membrane dynamics including sorting and trafficking (Wojtal et al., 2008). It has also been found to stimulate cell growth and has been implicated in cancer (Cho-Chung et al., 1995). Its role in transcription regulation occurs through phosphorylation of transcription factors of the cAMP response element binding (CREB) family. Activated CREB translocates to the nucleus where it binds to specific consensus promoter sequences resulting in the expression of over 4000 genes (Naviglio et al., 2009). PKA also plays a significant immunosuppressive role. In peripheral blood mononuclear cells (PBMCs), PKA has been implicated in increased synthesis of the anti-inflammatory cytokine interleukin-10 and decreased synthesis of tumor necrosis factor α (TNFα) under elevated cAMP conditions (Eigler et al., 1998). Specifically in monocytes, PKA activation has been linked to the negative regulation of cytokine and chemokine production, chemotaxis, phagocytosis and ROS production (Bryn et al., 2006). In T lymphocytes, PKA has been found to inhibit cytokine production, replication and proliferation (Aandahl et al., 2002; Skalhegg et al., 1992).

2. Protein Kinase A and Autophagy

Work done in yeast has identified a role for PKA in the regulation of autophagy (Budovskaya et al., 2005; Stephan et al., 2009; Yorimitsu et al., 2007). PKA-mediated autophagy inhibition in yeast has been found to occur through phosphorylation of Atg1 (yeast ortholog of ULK) and Atg13 (yeast ortholog of mAtg13), resulting in impairment of their localization to the PAS and decreased autophagy. The roles of cAMP and PKA in the regulation of mammalian cell autophagy are less clear. Both stimulation and inhibition of autophagy have been reported in mammalian cells upon treatment with cAMP or its analogs (Holen et al., 1991; Holen et al., 1996; Mavrakis et al., 2006; Seglen et al., 1991;
Shelburne et al., 1973). Negative regulation of autophagy by cAMP and PKA modulating drugs has been reported in rat hepatocytes (Holen et al., 1996). More recent work by Cherra et al. characterized a direct inhibitory role for PKA in autophagy through phosphorylation of LC3 (Cherra, III et al., 2010). This is the first time LC3 has been found to be phosphorylated therefore further work needs to be done in order to fully characterize this process. At the same time, another study has found a stimulatory role for cAMP in the induction of autophagy in rat livers (Shelburne et al., 1973). Due to these contradictory reports, the role and mechanism of function for cAMP and PKA in the regulation of mammalian autophagy remains to be fully characterized.
THESIS SUMMARY

In this thesis I identify and characterize novel roles for the second messengers diacylglycerol and cAMP in the regulation of autophagy. Autophagic targeting of S. Typhimurium has been previously shown by our lab to involve ubiquitin but only half of these targeted bacteria are positive for ubiquitin, indicating the existence of a second signalling mechanism for this pathway. I show that autophagy targeting of this non-ubiquitinated population requires the lipid second messenger diacylglycerol. I also show that diacylglycerol production at the bacteria-containing phagosome requires the actions of phospholipase D and phosphatidic acid phosphatase to convert phosphatidylcholine to diacylglycerol. Furthermore, I have identified that the diacylglycerol effector PKCd is required for autophagy of these bacteria. Finally, I show that the role for diacylglycerol and PKCd is not anti-bacterial autophagy specific but that both also play a role in rapamycin-induced autophagy. This is the first time that diacylglycerol has been implicated in regulation and targeting of autophagy and confirms a role for protein kinases in this process.

Bacteria employ strategies to avoid targeting by the autophagy pathway. This typically involves camouflaging themselves or delaying autophagosome maturation. To date, no bacterial species or factor has been identified that is able to inhibit autophagy on a cellular level. In the second data section of my thesis, I describe the mechanism by which two bacterial cAMP-elevating toxins, edema factor from B. anthracis and cholera toxin from V. cholerae are able to inhibit multiple types of autophagy. The role of cAMP and its effector PKA in mammalian autophagy has not been well characterized. Using pharmacological agents including PKA activators I show that both play a negative regulatory role in this process and that PKA can be hijacked by bacteria in order to inhibit this process.

Chapter 1 of this thesis contains a published review article (Shahnazari and Brumell, 2011). The first data chapter, Chapter 3 also contains a published article which I have used for the summary, data and discussion section (Shahnazari et al., 2010). Supplementary figures and data from this publication have also been incorporated into the data section.
Additionally, data from this chapter includes work done by Dr. Wei-lien Yen from Dr. Daniel Klionsky’s lab (University of Michigan) who contributed Table 3-2 and Figure 3-10. The second data chapter, Chapter 4 is comprised of an article that is currently in press. This article was used for the summary, data and discussion section of this chapter. Supplementary figures for this article have also been incorporated into the data section. Data in Figure 4-3A, B and C was generated with the help of Anton Namolovan and quantification of experiments in Figure 4-7 was performed by Dr. Peter Kim (University of Toronto). Chapter 2 contains the combined materials and methods sections from Chapter 3 and 4. Finally, the discussion section of this thesis is found in Chapter 5 which contains a figure and sections from a published article (Shahnazari et al., 2011).
CHAPTER 2
MATERIALS AND METHODS

Bacterial Strains and Cell Culture

Wild-type *S. Typhimurium* SL1344 (Hoiseth and Stocker, 1981), monomeric red fluorescent protein (mRFP)-expressing *S. Typhimurium* or *invA/inv S. Typhimurium* (Birmingham et al., 2006) were used for infection studies as indicated. *atg5*⁻/⁻ and *PKCδ*⁻/⁻ mouse embryonic fibroblasts (MEFs) have been previously described (Kuma et al., 2004; Miyamoto et al., 2002). HeLa (human epithelial), Henle-407 (human intestinal epithelial), and RAW 264.7 (murine macrophage) cell lines were obtained from the American Type Culture Collection (ATCC). HeLa, Henle, RAW 264.7 and mouse embryonic fibroblasts (MEFs) were maintained in Dulbecco’s modified Eagle’s growth medium (Thermo Scientific) supplemented with 10% fetal bovine serum (Wisent) at 37°C at 5% CO₂ without antibiotics. Cells were seeded in 24-well tissue culture plates (BD Biosciences) at 2.5 x 10⁴ cells/well 48 h prior to use for experiments involving direct visual analysis. Cells were seeded at 5.0 x 10⁴ cells/well 48 h prior to use for experiments involving siRNA treatment and Western blot analysis.

Bacterial Infection

Late-log *S. Typhimurium* cultures were used for infecting cells and prepared using a method optimized for bacterial invasion (Steele-Mortimer et al., 1999). Briefly, wild-type bacteria were grown in LB with antibiotics for approximately 16 h at 37°C with shaking and then diluted (1:33) in LB without antibiotics for 3 h. Bacterial inocula were prepared by pelleting at 10,000 x g for 2 min, diluted 1:100 in phosphate buffered saline (PBS), pH 7.2 and added to cells for 10 min at 37°C. After infection, extracellular bacteria were removed by extensive washing with PBS and addition of 100 μg/mL gentamicin to the growth medium at 30 min post-infection. For experiments requiring inhibition of bacterial protein synthesis, 200 μg/mL chloramphenicol was added directly to the growth medium after
infection and kept present throughout the rest of the infection. Infection with invA/inv S. Typhimurium has been previously described (Birmingham et al., 2006).

**Plasmids and Transfection**

For the transient transfection of cells with plasmid DNA, transfection reagents GeneJuice (EMD Biosciences), FuGene 6 (Roche Applied Sciences) and and ExGen 500 (Fermentas) were used according to the manufacturers’ instructions. Constructs used include: 3xmyc-Atg16L1 (Kuballa et al., 2008), GFP-LC3 (Kabeya et al., 2000), RFP-LC3 (kindly provided by Walter Beron, Universidad Nacional de Cuyo), PKCδ-C1-GFP (Tse et al., 2005), PH-PLCδ-GFP (Stauffer et al., 1998), 2FYVE-GFP (Vieira et al., 2001), GFP-PH-AKT (Weernink et al., 2000) and PMP34-GFP-Ub (G76V) (Kim et al., 2008). HA-PLD1 WT, HA-PLD2 WT, HA-PLD1 K898R and HA-PLD2 K758R were kindly provided by Michael Frohman (SUNY, New York). PKCδ expression plasmid – pMT2-PKCδ+/− was a kind gift from Michael Leitges (University of Oslo). DN JNK constructs, DN JNK1 (plasmid 13846) and DN JNK2 (plasmid 13761) (Derijard et al., 1994; Gupta et al., 1996) were obtained from Addgene. siRNA transfections were performed using Oligofectamine (Invitrogen) according to the manufacturer’s instructions.

**Phagocytosis assay**

Latex beads (3.87 μm, Bangs Laboratory) were opsonized for 16 h incubation at 4°C in 6 mg/mL human IgG (Biodesign International). Beads were sedimented onto cells (1,000 rpm for 1 min in a Beckman Coulter Allegra R6 centrifuge) and then placed at 37°C. Cells were washed with PBS after 15 min and fresh growth medium was added. Cells were fixed at the indicated time points and processed for immunofluorescence.
**Antibodies**

Primary antibodies used include rabbit polyclonal antibodies to S. Typhimurium O antiserum Group B (Difco Laboratories), PKCα and δ (Santa Cruz Biotechnology), Atg12 (Cell Signaling), YFP (Clontech), S133 phospho-CREB, CREB (Cell Signaling) and β-actin (Sigma). Rabbit polyclonal antibodies to LC3 (B-90) were a kind gift from Karla Kirkegaard (Stanford University). Rabbit polyclonal antibodies to p22 (R3179) were a kind gift from Mark T. Quinn (Montana State University). Mouse monoclonal antibodies used include those to mono- and polyubiquitinated conjugates, FK2 clone (Enzo Life Sciences), p62 (BD Biosciences) and β-tubulin (Sigma). All secondary antibodies used were Alexa Fluor conjugates (Molecular Probes).

**Pharmacological Agents**

Pharmacological agents used include D609, 6-bnz-cAMP, forskolin and KT5720, purchased from Enzo Life Sciences. DGK inhibitor I (also known as R59022), 3-methyladenine (3-MA), butanol, tert-butanol, phorbol 12-myristate 13-acetate (PMA), 4α-phorbol 12-myristate 13-acetate (4α-PMA), SP600125 and IBMX were purchased from Sigma. U73122, propranolol hydrochloride and rapamycin were purchased from Biomol International. Rottlerin and Go6983 were purchased from Calbiochem. siGENOME® SMARTpool® siRNA reagents (Dharmacon) were used for targeting human Atg12, Pap2B, p62, Pkca, Pkcd and p22 as well as scrambled control siRNA. Cholera toxin (Ctx; 101) was purchased from List Biological Laboratories, Inc. Protective antigen and Edema factor from *B. anthracis* were produced as previously described (Miller et al., 1999; Zhao et al., 1995).
**Immunofluorescence**

Cells were fixed with 2.5% paraformaldehyde in PBS, pH 7.2, for 10 min at 37°C. Fixed cells were permeabilized and blocked in 0.2% saponin (Calbiochem) and 10% normal goat serum (Wisent) for 2-12 h and stained as previously described (Brumell et al., 2001). Samples were mounted on slides using fluorescence mounting medium (Dako).

**Live Cell Imaging**

Alexa Fluor 647 carboxylic acid, succinimidyl ester (Invitrogen) was used to label wild-type *S. Typhimurium* SL1344 as previously described (Birmingham et al., 2006). HeLa cells were maintained at 37°C in RPMI (Thermo Scientific) during the course of the infection. A Leica DMIRE2 inverted fluorescence microscope equipped with a Hamamatsu Back-Thinned EM-CCD camera and spinning disk confocal scan head with a 63x objective and LSM 510 software was used. Volocity software (Improvision) was used to acquire images and perform deconvolution and 3D rendering.

**Confocal Microscopy and Image Preparation.**

All images shown are confocal z-slices taken using a Leica DMIRE2 inverted fluorescence microscope equipped with a Hamamatsu Back-Thinned EM-CCD camera and spinning disk confocal scan head with a 63x objective and LSM 510 software. Volocity software (Improvision) was used to acquire images. Confocal images were imported into Adobe® Photoshop™ and assembled in Adobe® Illustrator™. Colocalization and enumeration studies were performed on a Leica DMIRE2 fluorescence microscope by direct visualization.

**Yeast autophagy assays**
GFP-Atg8 processing was determined using wild-type (TN124), \textit{atg1Δ} (TYY127), \textit{pkc1}^{ts}, \textit{pkc1-3} and \textit{pkc1-4} cells expressing GFP-Atg8 as previously reported (Klionsky et al., 2007b). Briefly, cells were incubated in SD-N medium at the permissive (24°C) or non-permissive (38°C) temperature. Full-length GFP-Atg8 and free GFP were detected by Western blotting using anti-YFP antibodies. Bulk autophagic activity was determined using yeast strains expressing Pho8Δ60 as previously described (Klionsky et al., 2007b). Cells were grown in YPD at the permissive temperature. Half of the cell cultures were shifted to the non-permissive temperature for 30 min to inactivate Pkc1 while the other half remained at 24°C. Cells were then shifted to SD-N at either 24°C or 38°C for 2 h. Cell extracts were subsequently prepared for Pho8Δ60-dependent alkaline phosphatase activity measurements.

\textbf{Pexophagy analysis}

Fluorescent images were acquired with a Carl Zeiss LSM 710 laser-scanning confocal microscope and a 63x 1.4 NA Plan-Apochromat oil objective (Carl Zeiss MicroImaging, Inc.) All images were maximum intensity projection of seven optical slices in z-dimension. Each optical slice is 1 µm thick with 0.5 µm overlap between slices. Volocity software (Perkin Elmer) was used to calculate the number of peroxisomes in each cell using the z-stack images.

\textbf{Statistics.}

The mean ± s.e.m. for at least three independent experiments is shown in figures unless otherwise indicated, and \( p \) values were calculated using a two-tailed two-sample unequal variance Student’s \( t \)-test. A \( p \) value of less than 0.05 was determined to be statistically significant.
CHAPTER 3
A DIACYLGlycerol-DEPENDENT SIGNALING PATHWAY CONTRIBUTES TO REGULATION OF ANTI-BACTERIAL AUTOPHAGY

SUMMARY

The data from this chapter has been published as Shahnazari et al. Cell Host & Microbe 8(2): 137-146, (Shahnazari et al., 2010).

Autophagy mediates the degradation of cytoplasmic contents in the lysosome and plays a significant role in immunity. Lipid second messengers have previously been implicated in the regulation of autophagy, here we demonstrate a novel signaling role for diacylglycerol (DAG) in anti-bacterial autophagy. DAG production was necessary for efficient autophagy of Salmonella and its localization to bacteria-containing phagosomes preceded autophagy. The actions of phospholipase D and phosphatidic acid phosphatase were required for DAG generation and autophagy. Furthermore, the DAG-responsive δ isoform of protein kinase C was required as were its downstream targets JNK and NADPH oxidase. Previous studies have revealed a role for the ubiquitin binding adaptor molecules p62 and NDP52 in autophagy of S. Typhimurium. We observed bacteria-containing autophagosomes colocalizing individually with either DAG or ubiquitinated proteins, indicating that both signals can act independently to promote anti-bacterial autophagy. These findings reveal an important role for DAG-mediated PKC function in mammalian anti-bacterial autophagy.

RESULTS

Diacylglycerol associates with autophagy-targeted bacteria

Our lab and others have found that S. Typhimurium can be targeted by autophagy in a ubiquitin-dependent manner that requires the autophagy adaptors p62 (also called
SQSTM1) and NDP52 (Thurston et al., 2009; Zheng et al., 2009). These adaptors bind to ubiquitin and LC3 and serve to target ubiquitin-associated S. Typhimurium to the autophagy pathway (Thurston et al., 2009; Zheng et al., 2009). Knockdown of p62 and NDP52 expression leads to a partial, though not complete, decrease in autophagy of S. Typhimurium (Thurston et al., 2009; Zheng et al., 2009). In this regard, it is noteworthy that only 50% of S. Typhimurium targeted by autophagy (LC3+) are associated with ubiquitinated proteins (Birmingham et al., 2006), indicating the potential for a ubiquitin-independent pathway for autophagic targeting of bacteria prior to escape into the cytosol. We hypothesized that a lipid second messenger generated on SCV membranes mediates antibacterial autophagy.

We performed a screen for lipid second messengers associated with bacteria targeted by autophagy. Lipid second messengers were visualized by transfecting HeLa cells with fluorescent lipid-binding probes prior to infection (Table 3-1). The colocalization of each lipid with intracellular bacteria was assessed at 1 h post-infection (p.i.), the time of maximal autophagy of S. Typhimurium (Birmingham et al., 2006). To visualize SCVs targeted by autophagy we cotransfected cells with RFP fusions to LC3, a marker of autophagosomes (Klionsky et al., 2008). As an internal control, lipid colocalization with bacteria not targeted by autophagy (LC3-) was also quantified.

Using 2FYVE-GFP we monitored PI3P localization in S. Typhimurium-infected cells. We observed PI3P-containing puncta associated with LC3+ bacteria (Figure 3-1A). However, these puncta were also observed in association with LC3- bacteria, and there was no significant difference in the colocalization of PI3P with the two populations (Figure 3-1B). Production of PI3P by the class III PI3-kinase Vps34 is essential for autophagy in mammals and yeast, and PI3-kinase inhibitors block autophagy of S. Typhimurium (Kihara et al., 2001; Klionsky et al., 2008; Obara et al., 2006). Therefore, our findings suggest that PI3P production, although essential for autophagy, does not provide a localized signal that selects SCVs as cargo for autophagy. PI(4,5)P2 (PLCδ-PH-GFP) and PI(3,4)P2/PI(3,4,5)P3 (GFP-PH-AKT) were similarly found to be equally associated with both LC3+ and LC3- bacteria (Figure 3-1A, B).
Table 3-1 – Lipid probes used in this study.

<table>
<thead>
<tr>
<th>Lipid</th>
<th>Probe</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phosphatidylinositol 3-phosphate - PI(3)P</td>
<td>2FYVE-GFP</td>
<td>(Vieira et al., 2001)</td>
</tr>
<tr>
<td>Phosphatidylinositol 4,5-bisphosphate - PI(4,5)P₂</td>
<td>PH-PLCδ-GFP</td>
<td>(Stauffer et al., 1998)</td>
</tr>
<tr>
<td>Phosphatidylinositol 3,4-bisphosphate - PI(3,4)P₁</td>
<td>GFP-PH-AKT</td>
<td>(Weernink et al., 2000)</td>
</tr>
<tr>
<td>Phosphatidylinositol 3,4,5-bisphosphate - PI(3,4,5)P₃</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Diacylglycerol (DAG)</td>
<td>PKCδ-C1-GFP</td>
<td>(Tse et al., 2005)</td>
</tr>
</tbody>
</table>
An important lipid second messenger is DAG which induces translocation of responsive proteins to DAG-rich membranes (Carrasco and Merida, 2007). Using the C1 domain from PKCδ as a probe we visualized DAG. The probe localized to perinuclear transferrin and Rab5-positive endosomes in control (not infected) cells (Figure 3-2A). In S. Typhimurium-infected cells, DAG was preferentially found on LC3+ SCVs, suggesting that this lipid may play a role in autophagy (Figure 3-1A, B). The association of DAG with autophagy-targeted bacteria was further confirmed using antibodies to endogenous LC3 (Figure 3-2B). In fact, quantification revealed that endogenous LC3 preferentially colocalizes with DAG+ bacteria (Figure 3-2C). Another component of the autophagy machinery is Atg16L1 which acts as a targeting factor for localized induction of autophagy (Mizushima et al., 2003). Using a transiently transfected construct we also observed preferential colocalization of Atg16L1 with DAG+ bacteria (Figure 3-2D, E). These findings suggest that LC3 may be conjugated to DAG+ SCVs.

**DAG colocalization is independent of and precedes autophagy**

We also monitored the kinetics of DAG colocalization with the total population of intracellular bacteria (without scoring for LC3 colocalization). DAG association with SCVs peaked at 45 min p.i. (Figure 3-1C), prior to maximal autophagy of the bacteria which occurs at 1 h p.i. (Birmingham et al., 2006). Live cell imaging confirmed that recruitment of the DAG probe to individual SCVs precedes LC3 (Figure 3-3).

Previous studies have shown a requirement for bacterial protein synthesis after invasion and the bacterial SPI1-encoded T3SS for autophagy of S. Typhimurium (Birmingham et al., 2006). We therefore looked at DAG colocalization in the presence of the bacterial protein synthesis inhibitor chloramphenicol (CM) and observed an impairment in DAG recruitment (Figure 3-1C). S. Typhimurium normally invades cells by translocating effector proteins into host cells via the SPI1-T3SS. We examined the invA/inv mutant of S. Typhimurium which lack a functional SPI1 T3SS and are instead internalized through expression of the *Yersinia* Invasin protein (Steele-Mortimer et al., 2002). We have
Figure 3-1 – Diacylglycerol colocalizes with bacteria-containing autophagosomes.

(a) HeLa cells were co-transfected with RFP-LC3 and either 2FYVE-GFP (PI(3)P probe), PLCδ-PH-GFP (PI(4,5)P₂ probe), GFP-PH-AKT (PI(3,5)P₂ and PI(3,4,5)P₃ probe) or PKCδ-C1-GFP (DAG probe). Cells were infected with wild-type S. Typhimurium, fixed at 1 h p.i. and immunostained for S. Typhimurium. Representative confocal z-slices are shown. The inner panels represent a higher magnification of the boxed areas. Size bar, 10 µm. (b) The percentage of RFP-LC3⁺ or RFP-LC3⁻ bacteria colocalizing with the lipid probes in (a) was determined by fluorescence microscopy. (c) HeLa cells were transfected with PKCδ-C1-GFP and infected as in (a). Cells were treated with or without chloramphenicol (CM, 200 µg/mL) at 10 min p.i. for the remainder of the infection. Cells were fixed at the indicated time-points and immunostained for S. Typhimurium. The percentage of PKCδ-C1-GFP⁺ bacteria was enumerated by fluorescence microscopy. Data represent the mean ± standard error (s.e.m.) for three independent experiments.
Figure 3-2 – Characterization of the PKCδ-C1-GFP probe.

(a) HeLa cells were transfected with PKCδ-C1-GFP and either Rab5-RFP or immunostained for transferrin receptor, LAMP-1, ERGIC 53, GM130, Bip or Giantin. Transferrin was visualized using Alexa Fluor 546 conjugated transferrin. Size bar, 10 µm. (b) HeLa cells were transfected with PKCδ-C1-GFP, infected with wild-type S. Typhimurium, fixed at 1 h p.i. and immunostained for LC3 using a rabbit polyclonal antibody. Bacteria were immunostained using a rabbit polyclonal antibody in conjunction with a Zenon Alexa Fluor 647 labeling kit. Representative confocal z-slices are shown. Size bar, 10 µm. (c) Quantification of the colocalization of LC3 with DAG⁺ or DAG⁻ bacteria in b (d) HeLa cells were transfected with PKCδ-C1-GFP and 3xmyc-ATG16L1, infected with wild-type S. Typhimurium and fixed at 45 min p.i. Cells were immunostained for ATG16L1 using a mouse monoclonal antibody to myc and bacteria using a rabbit polyclonal antibody. Representative confocal z-slices are shown. Size bar, 10 µm. (e) Quantification of the colocalization of ATG16L1 with DAG⁺ or DAG⁻ bacteria in d. Data represent the mean ± standard error (s.e.m.) for three independent experiments.
previously shown that the invA/inv mutant is not targeted by autophagy (Birmingham et al., 2006). Here we observed that DAG did not colocalize with the invA/inv mutant (Figure 3-4A, B). These findings are consistent with a link between DAG production on SCVs and their targeting by autophagy.

To determine whether DAG is a signal for autophagy we next tested whether maintenance of the DAG signal would result in prolonged autophagy of S. Typhimurium at later time points p.i. Members of the DAG kinases (DGKs) act to attenuate DAG signaling levels by phosphorylating DAG to produce phosphatidic acid (PA) (Carrasco and Merida, 2007; Merida et al., 2008). Treatment with DGK inhibitor I (R59022) resulted in significantly more autophagy at 90 min p.i. when compared with vehicle-treated cells (Figure 3-4C, D). Therefore, decreased turnover of DAG maintains autophagic targeting of SCVs.

Since our data suggested a link between DAG and autophagy we wanted to determine whether DAG localization to bacteria was dependent on the cell’s autophagic machinery. It was reasonable to postulate that DAG accumulation on the SCV was the result of delivery of autophagic membrane. Indeed, the source(s) of membranes for autophagy and their lipid composition are not known (Mizushima, 2007). To address this question we examined wild-type and atg5/− (autophagy-deficient) mouse embryonic fibroblasts (MEFs). These cells were infected with S. Typhimurium, and bacterial colocalization with the DAG probe or LC3 was characterized in separate experiments. We determined that DAG colocalization to the SCV was independent of autophagy and still occurred in the absence of a functional autophagy system (in Atg5/− MEFs) (Figure 3-5A, B, C). We conclude that the DAG signal precedes autophagy and that its presence on SCVs is not due to delivery of autophagic membrane.

**DAG is generated by PAP and PLD and is required for autophagy**

Next, we examined the pathway of DAG generation on SCVs. In addition to de novo DAG biosynthesis, DAG generation can also occur via phospholipase C (PLC), phosphatidic acid phosphatase (PAP) and sphingomyelin synthase (SMS) (Figure 3-6A).
Figure 3-3 – Diacylglycerol colocalization to SCVs precedes LC3.

HeLa cells were co-transfected with PKCδ-C1-GFP and RFP-LC3, infected with wild-type S. Typhimurium stained with Alexa Fluor 647 carboxylic acid, succinimidyl ester (blue) and imaged over the course of the infection. Images corresponding with the indicated time points p.i. are shown.
Figure 3-4 – Diacylglycerol does not colocalize with invA/inv S. Typhimurium and maintenance of the signal prolongs autophagy.

(a-b) HeLa cells were transfected with PKCδ-C1-GFP, infected with invA/inv S. Typhimurium, fixed at the indicated time points and immunostained for the bacteria using a rabbit polyclonal antibody. One representative experiment is also shown for wild-type S. Typhimurium in b. Representative fluorescence images are provided for the 45 min p.i. time point. Data represent the mean ± standard error (s.e.m.) for three independent experiments for invA/inv S. Typhimurium invasion. (c) HeLa cells were transfected with GFP-LC3 and treated with either DMSO or DGK inhibitor I (R59022, 10 µM). Cells were infected with RFP-Sal and fixed at 90 min p.i. Representative confocal z-slices are shown. The inner panels represent a higher magnification of the boxed areas. Size bar, 10 µm. (d) HeLa cells were transfected, treated and infected as in c, and fixed at the indicated time points. The percentage of GFP-LC3+ bacteria was determined by fluorescence microscopy for each condition. Data represent the mean ± standard error (s.e.m.) for three independent experiments.
Treatment with the PLC inhibitor U73122 did not alter DAG or LC3 colocalization with S. Typhimurium, suggesting that PLC activity is not involved in autophagy (Figure 3-7A and Figure 3-6B). Additionally, treatment with the SMS inhibitor D609 did not have any effect on DAG colocalization with the bacteria (Figure 3-6C). In contrast, inhibition of PAP with propranolol hydrochloride resulted in significant decreases in both DAG colocalization with bacteria at 45 min p.i. and LC3 colocalization at 1 h p.i. (Figure 3-6B, C). siRNA to PAP also led to a significant decrease in LC3 colocalization with S. Typhimurium (Figure 3-6D and Figure 3-7B). These data demonstrate that DAG generation on SCVs occurs through the PAP pathway and that this pathway is required for anti-bacterial autophagy.

PAP activity requires a pool of PA substrate, which can be generated through the action of phospholipase D (PLD) (Figure 3-6A) (Carrasco and Merida, 2007). Inhibition of PLD activity with 1-butanol led to a consistent decrease in both DAG and LC3 colocalization with S. Typhimurium (Figure 3-7C, D). This effect was not observed with tert-butanol, an isomer of 1-butanol that does not inhibit PLD activity. Expression of catalytically inactive mutants of hPLD1 (K898R) and mPLD2 (K758R), which have been used previously as dominant negative constructs (DN) (Denmat-Ouisse et al., 2001), led to a significant decrease in autophagy of S. Typhimurium (Figure 3-6E and Figure 3-7E). Therefore, the generation of DAG on SCVs and subsequent autophagy of S. Typhimurium requires the action of both PAP and PLD.

PKC plays a conserved role in autophagy regulation

Classical and novel PKC isoforms contain DAG-binding C1 domains, and DAG binding has an impact upon their function (Griner and Kazanietz, 2007; Kazanietz, 2000). To test a role for PKCs in autophagy we utilized pharmacological PKC inhibitors. Treatment with rottlerin, but not Go6983 led to a significant decrease in autophagy of S. Typhimurium (Figure 3-8A, B). Neither inhibitor affected DAG levels on the SCV (Figure 3-8C, D). Rottlerin has affinity for PKCδ (Gschwendt et al., 1994). Therefore to further test a role for this PKC in anti-bacterial autophagy we used siRNA to target its expression (Figure 3-8E). Knockdown of
Figure 3-5 – Diacylglycerol production on SCVs is independent of autophagy.

(a-b) Wild-type (WT) and autophagy-deficient (atg5−/−) MEFs were transfected with either PKCδ-C1-GFP (a) or GFP-LC3 (b). Cells were infected with S. Typhimurium expressing mRFP (RFP-Sal). Cells were fixed at 45 min a or 1 h b p.i. Representative confocal z-slices are shown. The inner panels represent a higher magnification of the boxed areas. Size bar, 10 µm. (c) WT and atg5−/− MEFs were transfected and infected as in a-b and fixed at the indicated time-points. PKCδ-C1-GFP+ or GFP-LC3+ RFP-Sal were determined by fluorescence microscopy. Data represent the mean ± standard error (s.e.m.) for at least three independent experiments.
Figure 3-6 – Autophagy of *S. Typhimurium* requires phosphatidic acid phosphatase and phospholipase D.

(a) Pathways for the production of DAG. PC (phosphatidylcholine), PLD (phospholipase D), PA (phosphatidic acid), PAP (phosphatidic acid phosphatase), propr. (propranolol hydrochloride), PLC (phospholipase C), SMS (sphingomyelin synthase), SM (sphingomyelin).

(b) HeLa cells were transfected with GFP-LC3 and infected with RFP-*Sal*. Cells were treated with growth medium (GM), U73122 (10 µM), DMSO, propranolol hydrochloride (propr., 250 µM) or H₂O at 10 min p.i. for the remainder of the infection, and fixed at 1 h p.i. (c) HeLa cells were transfected with PKCδ-C1-GFP and infected with RFP-*Sal*. Cells were treated with GM, propranolol hydrochloride (propr., 250 µM) or D609 at the indicated concentrations at 10 min p.i. for the remainder of the infection, and fixed at 45 min p.i. (d) HeLa cells were co-transfected with GFP-LC3 and either control siRNA (si-CTRL) or siRNA specifically targeting Atg12 (si-ATG12) or PAP2B (si-PAP). Cells were infected with RFP-*Sal* and fixed at 1 h p.i. (e) HeLa cells were transfected with RFP-LC3 and either GFP, HA-PLD1 DN (dominant negative) or HA-PLD2 DN. Cells were infected with *S. Typhimurium*, fixed at 1 h p.i. and immunostained for *S. Typhimurium* and HA tag. The percentage of DAG⁺ or LC3⁺ *S. Typhimurium* was determined by fluorescence microscopy. Data represent the mean ± standard error (s.e.m.) for at least three independent experiments.
Figure 3-7 – DAG generation on SCVs requires phosphatidic acid phosphatase and phospholipase D.

(a) HeLa cells were transfected with PKCδ-C1-GFP and infected with RFP-Sal. Cells were treated with U73122 (10 µM), DMSO, propranolol hydrochloride (propr., 250 µM) or H2O at 10 min p.i. for the remainder of the infection, and fixed at 45 min p.i. (b) HeLa cells were co-transfected with GFP-LC3 and either control siRNA (si-CTRL) or siRNA specifically targeting Atg12 (si-ATG12) or PAP2B (si-PAP). Cells were infected with wild-type RFP-Sal and fixed at 1 h p.i. Representative confocal z-slices are shown. The inner panels represent a higher magnification of the boxed areas. (c-d) HeLa cells were transfected with either PKCδ-C1-GFP or GFP-LC3 and treated with 0.3% v/v 1-butanol (1-but) or tert-butanol (tert-but). Cells were infected with S. Typhimurium, fixed at 45 min c, or 1 h d p.i. and stained for S. Typhimurium. The number of PKCδ-C1-GFP^* c, or GFP-LC3^* d bacteria was determined for at least 100 bacteria for each condition. Values represent the percentage of marker-positive bacteria compared to control cells. (e) HeLa cells were transfected with RFP-LC3 and either GFP, HA-PLD1 DN (dominant negative) or HA-PLD2 DN. Cells were infected with S. Typhimurium, fixed at 1 h p.i. and immunostained for S. Typhimurium and HA tag. Representative confocal z-slices are shown. The inner panels represent a higher magnification of the boxed areas. Size bar, 10 µm. Data represent the mean ± standard error (s.e.m.) for at least three independent experiments.
PKCδ led to a significant decrease in autophagy of S. Typhimurium that was not observed upon siRNA targeting of PKCα expression (Figure 3-9A, B). The role for PKCδ in autophagy of S. Typhimurium was confirmed using PKCδ−/− MEFs (Figure 3-9C). Complementation of the PKCδ−/− MEFs with PKCδ expression from a plasmid restored autophagy to WT levels. To test whether PKCδ can restrict intracellular bacterial growth we also looked at bacterial replication in WT and PKCδ−/− MEFs and observed significantly more bacteria at 6 and 8 h p.i. in cells lacking PKCδ (Figure 3-9D) indicating that PKCδ plays a significant role in anti-bacterial autophagy.

*Saccharomyces cerevisiae* (*S. cerevisiae*) contains a single PKC isozyme, Pkc1. Using temperature sensitive (ts) mutants of this kinase (Table 3-2) we examined its role in starvation-induced autophagy. In yeast, autophagosomes are delivered to the vacuole whereupon their contents are degraded. We used yeast expressing GFP-Atg8, which upon delivery to the vacuole via autophagosomes is degraded to produce free GFP. We found that *pkc1* mutants (*pkc1*<sup>ts</sup>, *pkc1*-3 and *pkc1*-4) were defective in GFP-Atg8 processing in starvation-induced autophagy at the non-permissive temperature (NPT, 38°C) compared to the permissive temperature (PT, 24°C) (Figure 3-10A). As expected, cells lacking Atg1 (essential for autophagy) did not display GFP-Atg8 processing. We also measured alkaline phosphatase (Pho8Δ60) activity during starvation-induced autophagy. Pho8Δ60 is a truncation of the vacuolar alkaline phosphatase, Pho8, and is expressed in an inactive form that matures only upon delivery to the vacuole by autophagy (Noda et al., 1995). Pho8Δ60 activity was compromised in *pkc1* mutants, indicating a required role for this kinase in starvation induced-autophagy in yeast (Figure 3-10B). Therefore, PKC plays a conserved role in autophagy regulation.

Next, we addressed the mechanism by which PKCδ regulates autophagy in mammalian cells. Previously, it was shown that PKCδ regulates the interaction between Bcl-2 and Beclin 1 via c-Jun N-terminal kinase (JNK) activation (Chen et al., 2008). Therefore, we treated cells with the JNK inhibitor SP600125 and observed impairment of anti-bacterial
a. Immunofluorescence images showing the expression of RFP-Sal, GFP-LC3, and their merge under different conditions.

b. Bar graph showing the percentage of LC3-positive Sal with statistical significance.

c. Immunofluorescence images showing the expression of RFP-Sal, PKCδ1-C1-GFP, and their merge under different conditions.

d. Bar graph showing the percentage of DAG-positive Sal.

e. Western blot analysis showing the expression levels of Atg12, PKCα, PKCδ, and β-Actin under different conditions.
Figure 3-8 – Protein kinase C is required for autophagy of *S. Typhimurium*.

HeLa cells were transfected with GFP-LC3 (a) or PKCδ-C1-GFP (c) and treated with either DMSO, rottlerin (15 μM) or Go6983 (10 nM). Cells were infected with RFP-*Sal* and fixed at 1 h a, or 45 min c p.i. Representative confocal z-slices are shown. The inner panels represent a higher magnification of the boxed areas. Size bar, 10 μm. (b) HeLa cells were transfected and treated as in a. The percentage of GFP-LC3⁺ bacteria was determined by fluorescence microscopy. (d) HeLa cells were transfected and treated as in c. The percentage of PKCδ-C1-GFP⁺ was determined by fluorescence microscopy. Data represent the mean ± standard error (s.e.m.) for at least three independent experiments. (e) HeLa cells were transfected with either control siRNA (si-CTRL) or siRNA specifically targeting Atg12 (si-ATG12), PKCδ (si-PKCδ) or PKCα (si-PKCα). Western blotting was used to detect protein levels of Atg12, PKCα, PKCδ and β–actin using anti-Atg12, anti-PKCα, anti-PKCδ and anti-β–actin (as a loading control) antibodies, respectively.
**Table 3-2 – Yeast strains used in this study.**

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**Figure 3-9 – Autophagy of S. Typhimurium requires protein kinase C.**

(a-b) HeLa cells were co-transfected with GFP-LC3 and either control siRNA (si-CTRL) or siRNA specifically targeting Atg12 (si-ATG12), PKCδ (si-PKCδ) or PKCα (si-PKCα). Cells were infected with wild-type RFP-Sal and fixed at 1 h p.i. Representative confocal z-slices are shown a. The inner panels represent a higher magnification of the boxed areas. Size bar represents 10 μm. The percentage of GFP-LC3⁺ bacteria was determined by fluorescence microscopy b. (c) WT and PKCδ-deficient (PKCδ⁻/⁻) MEFs were transfected with GFP-LC3 and/or a PKCδ expression plasmid. Cells were infected with RFP-Sal and fixed at 1 h p.i. The percentage of GFP-LC3⁺ bacteria was determined by fluorescence microscopy. (d) WT and PKCδ-deficient (PKCδ⁻/⁻) MEFs were infected with wild-type S. Typhimurim and lysed at the indicated time points. The number of bacteria was determined by the number of colonies formed on agar plates. Data represent the mean ± standard error (s.e.m.) for at least three independent experiments.
autophagy (Figure 3-11A). This role was further confirmed using DN constructs (Figure 3-11B). PKCδ also activates NADPH oxidases (Fontayne et al., 2002; Pendyala et al., 2008) which we have previously shown to be required for anti-bacterial autophagy (Huang et al., 2009). siRNA knockdown of p22phox, an essential component of NOX1-4 NADPH oxidases (Nauseef, 2008) impaired autophagy of Salmonella but had no effect on DAG localization to the bacteria (Figure 3-11C, D, E). Therefore, DAG localization to the SCV is not sufficient to promote autophagy of Salmonella in the absence of NADPH oxidase activity. These studies suggest that PKCδ promotes autophagy of bacteria through JNK and NADPH oxidase activation.

**DAG and ubiquitin function independently to induce anti-bacterial autophagy**

Having characterized DAG as a novel signal required for autophagy of S. Typhimurium, the possibility exists that it may act in conjunction with ubiquitin-dependent autophagy adaptors (p62/NDP52) in order to target bacteria to this pathway. To test this possibility we cotransfected HeLa cells with the DAG probe and RFP-LC3 and immunostained for endogenous ubiquitin (mono- and polyubiquitinated proteins). We examined LC3+ S. Typhimurium and were able to observe bacteria colocalizing individually with either DAG or ubiquitin (Figure 3-12A). We quantified colocalization of ubiquitin with DAG+ bacteria at 45 and 60 min p.i. and observed that the majority of DAG+ bacteria were negative for ubiquitin (~65% at both time points) (Figure 3-12B). We also quantified the colocalization of p62 with DAG+ and DAG- S. Typhimurium populations and observed that p62 colocalized significantly more with DAG- bacteria (Figure 3-12C). Additionally, we targeted components of each pathway (DAG and p62) separately and in combination in order to determine the contribution of each pathway to anti-bacterial autophagy. Using siRNA and pharmacological agents we observed additive inhibitory effects in the presence of siRNA to p62/PAP and p62/with rottlerin treatment (Figure 3-12D and Figure 3-11F). We also observed additive inhibitory effects in the presence of siRNA to p62/PKCδ although this
### a

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### b

![Comparison of Pho8Δ60 activity](image)

- SMD PT
- SD-N PT
- SD-N NPT

- **WT**: Bar with height indicating % Pho8Δ60 activity (e.g., 100%)
- **atg1**: Bar with height indicating % Pho8Δ60 activity (e.g., 80%)
- **pkc1 ts**: Bar with height indicating % Pho8Δ60 activity (e.g., 60%)
- **pkc1-3**: Bar with height indicating % Pho8Δ60 activity (e.g., 40%)
- **pkc1-4**: Bar with height indicating % Pho8Δ60 activity (e.g., 20%)

- **P < 0.001**
- **P < 0.05**
- **P = 0.001**
Figure 3-10 – Pkc1 is required for starvation-induced autophagy in yeast.

(a) GFP-Atg8 processing was monitored in wild-type (TN124), atg1Δ (TYY127), pkc1ts, pkc1-3 and pkc1-4 cells expressing GFP-Atg8. Cells were grown in SD-N medium at the permissive (PT, 24˚C) or non-permissive (NPT, 38˚C) temperature. Full-length GFP-Atg8 and free GFP were detected by Western blotting using anti-YFP antibodies. (b) Autophagic activity was determined by obtaining extracts from wild-type (TN124), atg1Δ (TYY127), pkc1ts, pkc1-3 and pkc1-4 S. cerevisiae expressing Pho8Δ60 and analyzed for Pho8Δ60-dependent alkaline phosphatase activity. Synthetic defined media lacking nitrogen (SD-N). Data represent the mean ± standard error (s.e.m.) for at least three independent experiments.
Figure 3-11 – JNK and NADPH oxidases are required for autophagy of S. Typhimurium

(a) HeLa cells were transfected with GFP-LC3 and infected with RFP-Sal. Cells were treated with DMSO or 5 µM SP600125 and fixed at 1 h p.i. (b) HeLa cells were transfected with GFP-LC3 and either DN JNK1, DN JNK2 or both, infected with RFP-Sal and fixed at 1 h p.i. The percentage of GFP-LC3⁺ bacteria in a and b was determined by fluorescence microscopy. (c-d) Henle cells were co-transfected with either control siRNA (si-CTRL) or siRNA specifically targeting Atg12 (si-ATG12) or p22 (si-p22) and GFP-LC3 c or PKCδ-C1-GFP d. Cells were infected with RFP-Sal and fixed at 1 h c, or 45 min d p.i. The percentage of GFP-LC3⁺ c and PKCδ-C1-GFP⁺ d bacteria was determined by fluorescence microscopy. Data represent the mean ± standard error (s.e.m.) for at least three independent experiments. (e) Western blotting was used to detect protein levels of p22 and β–actin in the presence of si-CTRL or si-p22 using anti-p22 and anti-β–actin (as a loading control) antibodies. (f) Western blotting was used to detect protein levels of p62 and β–actin in the presence of si-CTRL or si-p62 using anti-p62 and anti-β–actin (as a loading control) antibodies.
Figure 3-12 – DAG and p62 act in independent signalling pathways for anti-bacterial autophagy. (a) HeLa cells were co-transfected with PKCδ-C1-GFP and RFP-LC3. Cells were infected with wild-type S. Typhimurium, fixed at 45 min p.i. and immunostained for S. Typhimurium and mono- and polyubiquitinated proteins. Representative confocal z-slices are shown. The inner panels represent a higher magnification of the boxed areas. Size bar, 10 µm. (b) HeLa cells were transfected with PKCδ-C1-GFP, infected with wild-type S. Typhimurium and fixed at 45 and 60 min p.i.. Cells were immunostained for ubiquitinated protein and bacteria as in a. DAG+ bacteria were scored for colocalization with ubiquitin. (c) HeLa cells were transfected with PKCδ-C1-GFP, infected with wild-type S. Typhimurium, fixed at 45 min p.i. and immunostained for S. Typhimurium and p62. p62 colocalization was quantified for DAG+ and DAG- bacteria. (d) HeLa cells were co-transfected with GFP-LC3 and either control siRNA (si-CTRL) or siRNA specifically targeting PKCδ (si-PKCδ), PAP (si-PAP) or p62 (si-p62). Cells were infected with wild-type S. Typhimurium and treated with 15 µM rottlerin where indicated. Cells were fixed at 1 h p.i. and quantified for colocalization with GFP-LC3. Data represent the mean ± standard error (s.e.m.) for three independent experiments.
effect was not statistically significant. These data are consistent with a model whereby both signals (DAG and ubiquitin) can act independently as inducers of anti-bacterial autophagy.

**DISCUSSION**

Selective autophagy is categorized based upon the cargo, which can include bacteria (xenophagy), mitochondria (mitophagy), peroxisomes (pexophagy) and other cytoplasmic contents. The mechanisms by which different cargo are specifically targeted for degradation by autophagy have remained unclear. Recent work in anti-bacterial autophagy of *S. Typhimurium* has characterized a role for protein ubiquitination in the targeted degradation of bacteria (Thurston et al., 2009; Zheng et al., 2009). This process is mediated by at least two different autophagy adaptors: p62 and NDP52, that bridge the ubiquitin and autophagy pathways and thereby restrict bacterial growth (Thurston et al., 2009; Zheng et al., 2009). A ubiquitin-dependent pathway involving p62 also contributes to autophagy of *Listeria monocytogenes* (Yoshikawa et al., 2009) and disrupted vacuolar membrane associated with *Shigella flexneri* (Dupont et al., 2009). Despite the recognized importance of ubiquitin-dependent autophagy of bacteria, we have previously shown that only ~50% of *S. Typhimurium* targeted by autophagy (LC3+) colocalize with ubiquitinated proteins, suggesting the presence of an alternative ubiquitin-independent pathway (Birmingham et al., 2006). Consistent with this notion, we recently observed that depletion of p62 only partially inhibits autophagy of *S. Typhimurium* (Zheng et al., 2009).

Here we provide several lines of evidence indicating that DAG can also serve as a signal to promote anti-bacterial autophagy: i) kinetic analysis and live cell imaging demonstrated that DAG is localized to bacteria prior to LC3 recruitment, ii) DAG colocalization with bacteria was independent of Atg5, indicating that DAG was not recruited to bacteria with autophagic membranes, iii) inhibition of DAG formation with genetic or pharmacological approaches impaired autophagy of bacteria, and iv) DAG was present on SCVs that did not colocalize with ubiquitin, and inhibition of both DAG and p62 pathways resulted in an additive inhibitory effect on anti-bacterial autophagy. Therefore, we propose
Figure 3-13 – Two signals target *S. Typhimurium* to the autophagy pathway.

Model depicting the dual pathways by which *S. Typhimurium* is targeted by autophagy in mammals and the conserved role for PKC in the regulation of this process in both mammals and yeast. Data is consistent with two independent pathways for induction of antibacterial autophagy in mammalian cells (DAG and ubiquitin pathways). Dashed line and question mark between pathways represents the possibility that the two pathways may interact with each other.
that DAG-dependent and ubiquitin-dependent pathways contribute independently to autophagy of *S. Typhimurium* (Figure 3-13). We do not rule out the possibility that these pathways can interact and have overlapping impacts on autophagy of bacteria. It is also expected that the relative activity of these pathways will depend on the cell type examined and the virulence factors employed by the pathogen. Importantly, our data suggest that multiple pathways contribute to activation of anti-bacterial autophagy, providing a level of redundancy for innate immune defense.

Much remains to be learned about these two signalling pathways. For example, with ubiquitin-dependent autophagy it is not clear which proteins, bacterial or eukaryotic, are ubiquitinated to recruit the cytosolic autophagy adaptors. Similarly, the E3 ligases that mediate these ubiquitination events to initiate autophagy have not been identified. It is noteworthy that both *S. Typhimurium* and *S. flexneri* encode ubiquitin E3 ligases that are translocated into the host cell by type III secretion systems during the early stages of infection (Quezada et al., 2009; Rohde et al., 2007a; Rytkonen and Holden, 2007). Thus, it is tempting to speculate that bacterial E3 ligases promote anti-bacterial autophagy. Indeed, LaFont and colleagues have suggested the possibility that these bacteria initiate ubiquitin-dependent autophagy as a mechanism to suppress pro-inflammatory/cytotoxic signaling events localized to disrupted phagosomes (Dupont et al., 2009).

Whether DAG-dependent autophagy is a bacterial or host initiated process is unclear. Localization of DAG to *S. Typhimurium* required the SPI1 T3SS. Consistent with this observation, we previously found that the SPI1 T3SS is also required for autophagy of *S. Typhimurium* (Birmingham et al., 2006). Membrane damage may provide a signal for DAG production and autophagy. The pore-forming translocon component of type III secretion systems causes membrane damage in eukaryotic cells, including disruption of SCVs during *S. Typhimurium* infection (Birmingham et al., 2006; Perrin et al., 2004; Roy et al., 2004; Veenendaal et al., 2007). Membrane damage has also been associated with autophagic targeting of latex bead phagosomes following their entry into non-phagocytic cells via coating with lipid-based transfection reagents (Kobayashi et al., 2010). Osmotic swelling is another method to induce endosomal rupture that may be analogous to bacterial type III
Figure 3-14 – Diacylglycerol, phosphatidic acid phosphatase and phospholipase D are required for rapamycin-induced autophagy

(a) HeLa cells were co-transfected with RFP-LC3 and either 2FYVE-GFP (PI(3)P probe), PLCδ-PH-GFP (PI(4,5)P₂ probe), GFP-PH-AKT (PI(3,5)P₂ and PI(3,4,5)P₃ probe) or PKCδ-C1-GFP (DAG probe). Cells were treated with rapamycin (25 µg/mL) and fixed 2 h post-treatment. Representative confocal z-slices are shown. The inner panels represent a higher magnification of the boxed areas. Size bar, 10 µm. (b) The percentage of autophagosomes colocalizing with the lipid probes in a was determined by fluorescence microscopy. (c-d) HeLa cells were co-transfected with GFP-LC3 and either control siRNA (si-CTRL) or siRNA specifically targeting Atg12 (si-ATG12) or PAP2B (si-PAP). Cells were treated with rapamycin (25 µg/mL) and fixed at 2 h post-treatment. 100 cells were counted and scored for autophagosomes/cell via fluorescence microscopy. Representative confocal z-slices are shown in d. Arrowheads represent autophagosomes. Size bar, 10 µm. (e-f) HeLa cells were transfected with either control siRNA (si-CTRL) or siRNA specifically targeting Atg12 (si-ATG12), PKCδ (si-PKCδ) or PKCα (si-PKCα). Cells were treated with rapamycin (25 µg/mL) and fixed at 2 h post-treatment. 100 cells were counted and scored for autophagosomes/cell via fluorescence microscopy. Representative confocal z-slices are shown f. Arrowheads indicate autophagosomes and size bar, 10 µm. Data represent the mean ± standard error (s.e.m.) for at least three independent experiments.
secretion or other toxin damage. Interestingly, DAG is generated on endosomes recovering from osmotic shock (Shaughnessy et al., 2007), consistent with a role for autophagy in repairing damaged endosomes (Birmingham et al., 2006; Kobayashi et al., 2010). We have shown that the activity of PLD and PAP are required for DAG localization to bacteria and contribute to anti-bacterial autophagy (Figure 3-13). The nature of the upstream signals that lead to activation of these enzymes remains unclear, and will be the subject of future study. While we favor the model of membrane damage inducing autophagy, it is possible that S. Typhimurium translocates one or more type III secreted effector proteins into host cells to promote DAG production on SCVs and subsequent autophagy, analogous to the proposed initiation of autophagy by translocated E3 ligases.

How does DAG promote autophagy of S. Typhimurium? We propose that DAG acts at the target organelle (SCV) to recruit the DAG-binding PKC isoform PKCδ which can activate autophagy via the JNK and NADPH oxidase pathways. Interestingly, we also observed DAG on rapamycin-induced autophagosomes (Figure 3-14A, B). Other lipid-binding fluorescent probes were not significantly associated with these autophagosomes (Figure 3-14A, B). We also found that inhibition of PAP and PKCδ using an siRNA strategy resulted in significant inhibition of this type of autophagy (Figure 3-14C, D, E, F). These observations suggest a role for DAG/PKC in other types of autophagy. In fact, other DAG-responsive PKC isoforms (δ and θ) have also been implicated in hypoxia and ER stress-induced autophagy, respectively (Chen et al., 2008; Sakaki et al., 2008). However, it remains to be determined whether DAG plays a role in these forms of autophagy. We find that Pkc1 plays an important role in yeast autophagy, further suggesting a model wherein PKC isoforms are central and evolutionarily conserved regulators of autophagy. In mammalian cells it can be postulated that novel PKC isoforms are recruited and activated in a DAG-dependent manner to cargo selected for autophagy. The involvement of multiple PKC isoforms in autophagy regulation in mammals is reminiscent of Atg1, an essential autophagic factor whose regulatory role is conserved but has become more complex in higher eukaryotes (Chan and Tooze, 2009). To date, over 13 types of autophagy have been described in eukaryotes (Klionsky et al., 2007a). It remains to be seen whether DAG and
PKC isoforms play a role in these processes and whether targeted modulation of their activities can provide therapeutic benefits for treatment of diseases where autophagy has been implicated in pathogenesis.
CHAPTER 4
BACTERIAL TOXINS CAN INHIBIT HOST CELL AUTOPHAGY THROUGH cAMP ELEVATION IN HOST CELLS

SUMMARY

The data from this chapter is currently in press as Shahnazari et al. Autophagy 7(9).

Autophagy plays a significant role in innate and adaptive immune responses to microbial infection. Some pathogenic bacteria have developed strategies to evade killing by host autophagy. These include the use of ‘camouflage’ proteins to block targeting to the autophagy pathway and the use of pore-forming toxins to block autophagosome maturation. However, general inhibition of host autophagy by bacterial pathogens has not been observed to date. Here we demonstrate that bacterial cAMP-elevating toxins from B. anthracis and V. cholera can inhibit host antimicrobial autophagy, including autophagic targeting of S. Typhimurium and latex bead phagosomes. Autophagy inhibition required the cAMP effector protein kinase A. Formation of autophagosomes in response to rapamycin and the endogenous turnover of peroxisomes was also inhibited by cAMP-elevating toxins. These findings demonstrate that cAMP-elevating toxins, representing a large group of bacterial virulence factors, can inhibit host autophagy to suppress immune responses and modulate host cell physiology.

RESULTS

cAMP-elevating toxins activate PKA

Both stimulation and inhibition of autophagy have been reported in mammalian cells upon treatment with cAMP or its analogs (Holen et al., 1991; Holen et al., 1996; Mavrakis et al., 2006; Seglen et al., 1991; Shelburne et al., 1973). We initially hypothesized
that bacterial cAMP-elevating toxins can act as repressors of autophagy. To test this possibility, we chose two distinct bacterial cAMP-elevating toxins to study. Edema toxin (Edtx) from *Bacillus anthracis*, is an AB toxin comprised of the catalytic A component, edema factor (EF), and the B component protective antigen (PrA), which is required for delivery of EF to the mammalian cytosol. EF itself is a calmodulin-dependent AC able to directly increase intracellular cAMP levels. We also tested cholera toxin (Ctx) from *Vibrio cholera*, another AB toxin. Its A component functions as an ADP ribosyltransferase capable of indirectly increasing intracellular cAMP levels through activation of Gαs and as a result host ACs.

PKA is a holoenzyme consisting of two regulatory and two catalytic subunits. An increase in intracellular cAMP levels results in cAMP binding to the regulatory subunits resulting in release of the catalytic subunits. The free catalytic subunits of PKA can phosphorylate cAMP-response element binding protein (CREB) at its serine 133 (S133) residue (Sands and Palmer, 2008). Therefore, in order to ensure that the bacterial toxins Edtx and Ctx were functional and activating PKA, we performed Western blot analysis probing for S133 phospho-CREB. Elevated levels of phospho-CREB were observed in human intestinal epithelial cells, Henle-407 (Figure 4-1A) as well as murine RAW 264.7 macrophages (Figure 4-1B) upon treatment with Edtx and Ctx.

cAMP-elevating toxins can inhibit FcγR-mediated autophagy

We and others have observed that stimulation of immunity related cell surface receptors can induce the recruitment of LC3 to phagosomes (Huang et al., 2009; Sanjuan et al., 2007). LC3 localization to the phagosome has been shown to result in enhanced phagosome maturation (Sanjuan et al., 2007). We tested whether cAMP-elevating toxins have an effect on Fcγ receptor (FcγR)-mediated autophagic targeting of human IgG-opsonized latex beads in RAW 264.7 murine macrophages. Cells were transiently transfected with GFP-LC3, pre-treated with toxin and incubated with beads for 1 h prior to
Figure 4-1 – Edema toxin and Cholera toxin activate PKA in Henle-407 and RAW 264.7 macrophages.

(a) Henle-407 cells were treated with PrA (5 µg/mL), EF (10 µg/mL) or Ctx (1 µg/mL) for 1h. (b) RAW 264.7 macrophages were treated with PrA (10 µg/mL), EF (10 µg/mL) or Ctx (5 µg/mL) for 2 h. (a,b) Cells were harvested and cell lysates analysed by Western blotting. Antibodies used include those against phospho CREB (serine 133), CREB and β-tubulin.
Figure 4-2 – cAMP-elevating toxins can inhibit FcγR-mediated autophagy.

(a,b,c) RAW 264.7 macrophages were transiently transfected with GFP-LC3 and pretreated with growth medium (GM), (a,b) PrA (10 µg/mL) and/or EF (10 µg/mL) or (a,c) Ctx (5 µg/mL) 1 h prior to incubation with human IgG opsonised latex beads. Cells were fixed 1 h post-incubation. Extracellular beads were immunostained and not quantified. (a) Representative images are shown for each condition. Arrowheads indicate internalized beads, size bar represents 10 µm. (b,c) Quantification of LC3⁺ beads was performed for at least 100 internalized beads by fluorescence microscopy. Data represents the mean ± SEM for three independent experiments, * indicates $P \leq 0.01$. 
fixation. Extracellular beads were identified with immunostaining and excluded from analysis. We observed that pre-treatment of cells with Edtx resulted in a significant decrease in LC3 recruitment to phagosomes containing these beads (Figure 4-2A, B) while treatment with PrA or EF alone did not have an effect. Similarly, pre-treatment with Ctx resulted in a significant decrease in LC3 localization to latex-bead containing phagosomes (Figure 4-2A, C).

**cAMP-elevating toxins inhibit autophagy of S. Typhimurium and increase intracellular bacterial replication**

Many bacterial species are known to be targeted for degradation by the autophagy pathway, including *S. Typhimurium*. We have previously shown that autophagy of *S. Typhimurium* limits intracellular replication of these bacteria (Birmingham et al., 2006; Shahnazari et al., 2010; Zheng et al., 2009). We therefore tested the effect of treatment with Edtx and Ctx on autophagy of these bacteria. Henle-407 human intestinal epithelial cells were pre-treated with toxin, infected with RFP expressing *S. Typhimurium* (RFP-*Sal*) and fixed at 1 h post-infection (p.i.), the timepoint of maximal autophagy of *S. Typhimurium* (Birmingham et al., 2006). We observed that treatment with Edtx but not PrA or EF alone, resulted in a significant decrease in autophagy of *S. Typhimurium* (Figure 4-3A, B and Figure 4-4). Similarly, treatment with Ctx resulted in a significant decrease in antibacterial autophagy (Figure 4-3A, C).

Autophagy has been found to restrict the intracellular replication of *S. Typhimurium* (Birmingham et al., 2006; Shahnazari et al., 2010; Zheng et al., 2009). To test whether cAMP-mediated autophagy inhibition regulates bacterial infection, we quantified intracellular bacterial numbers in cells infected in the presence of Edtx or Ctx. At 2 h p.i., the majority of infected cells treated with growth medium, Edtx or Ctx contained between 1-5 bacteria (Figure 4-3D). At 10 h p.i., we observed a significantly higher percentage of cells containing 20 or more bacteria when treated with Edtx or Ctx compared with control
Figure 4-3 – cAMP-elevating toxins inhibit autophagy of S. Typhimurium and increase intracellular bacterial replication.

(a-c) Henle-407 cells were transiently transfected with GFP-LC3 and pre-treated with growth medium (GM), (a,b) PrA (5 µg/mL) and/or EF (10 µg/mL) or (a,c) Ctx (1 µg/mL) 1 h prior to invasion with RFP-Sal. Infected cells were fixed at 1 h post-infection. (a) Representative images are shown for each condition; inner panels represent a higher magnification of the boxed area, size bar represents 10 µm. (b,c) Quantification of LC3+ bacteria was performed by fluorescence microscopy for at least 100 bacteria. Data represents the mean ± SEM for three independent experiments. (d) Henle-407 cells were pretreated with growth medium (GM), PrA (5 µg/mL) and/or EF (10 µg/mL) or Ctx (1 µg/mL) 1 h prior to invasion with RFP-Sal. Cells were maintained in the indicated conditions during invasion and were fixed at 2h or 10 h post-infection. Bacterial number per infected cell was quantified by fluorescence microscopy and categorized into five categories (1-5, 6-10, 11-15, 16-20 and >20) for at least 100 cells. The percentage of infected cells for each category was quantified and compared with control conditions. Data represents the mean ± SEM for three independent experiments, * indicates $P < 0.05$ and ** indicates $P < 0.01$. 
Figure 4-4 – Edema toxin and Cholera toxin inhibit antibacterial autophagy.

Henle-407 cells were transiently transfected with GFP-LC3 and pre-treated with PrA (5 µg/mL) or EF (10 µg/mL) 1 h prior to invasion with RFP-Sal. Infected cells were fixed at 1 h post-infection. Representative images are shown; inner panels represent a higher magnification of the boxed area, size bar represents 10 µm.
conditions. These results indicate that cAMP-elevating bacterial toxins can inhibit antibacterial autophagy resulting in increased intracellular bacterial replication.

**cAMP-elevating drugs can inhibit FcγR-mediated and antibacterial autophagy**

In order to confirm that the cAMP-elevating toxins are acting through cAMP and to test the possibility that the cAMP effector PKA contributes to autophagy inhibition, we tested whether cAMP-elevating or PKA-activating pharmacological agents could phenocopy the results observed with cAMP-elevating toxins. Forskolin, an activator of host adenylate cyclases and IBMX an inhibitor of phosphodiesterases which convert cAMP to AMP, both act to increase intracellular cAMP levels (Beavo et al., 1970; Seamon et al., 1981). 6-bnz-cAMP is a cAMP mimetic capable of specifically activating PKA (Christensen et al., 2003). These agents were used to examine antimicrobial autophagy.

We initially tested a role for these drugs in FcγR-mediated autophagy and observed a significant decrease in LC3 recruitment to bead-containing phagosomes upon pre-treatment with either 6-bnz-cAMP, IBMX or forskolin (Figure 4-5A, B). In order to test a role for PKA in this process we co-treated cells with KT5720, an inhibitor of the catalytic subunit of PKA (Kase et al., 1987). We found that co-treatment of 6-bnz-cAMP, forskolin and IBMX with KT-5720 reversed the inhibitory effect of these PKA-activating drugs on LC3 recruitment to phagosomes (Figure 4-5B). This indicates that cAMP elevation is sufficient to inhibit autophagy and that this process is PKA-dependent. To confirm a role for PKA in toxin-mediated inhibition of this process we tested the effect of co-treatment of toxins with KT5720. We found that co-treatment of Edtx or Ctx with KT5720 blocked the inhibition of LC3 recruitment to phagosomes observed in the absence of this PKA inhibitor (Figure 4-5C).

Having found that treatment with Edtx and Ctx results in significantly increased bacterial replication, we were interested in determining whether cAMP-elevating pharmacological agents would also have this effect. Similar to that observed with the toxins, the majority of infected cells treated with vehicle, 6-bnz-cAMP, forskolin or IBMX contained between 1-5 bacteria at 2 h p.i. (Figure 4-5D). However, at 10 h p.i., we found
Figure 4-5 – cAMP-elevating drugs can inhibit FcγR-mediated and antibacterial autophagy.

(a,b) RAW 264.7 macrophages were transiently transfected with GFP-LC3 and pretreated with growth medium (GM), 6-bnz-cAMP (500 µM), IBMX (10 µM), forskolin (100 µM) and/or KT5720 (5 µM) where indicated, 1 h prior to incubation with human IgG opsonised latex beads. Cells were fixed 1 h post-incubation. Extracellular beads were immunostained and not quantified. (a) Representative images are shown for each condition. Arrowheads indicate internalized beads, size bar represents 10 µm. (b) Quantification of LC3+ beads was performed for at least 100 internalized beads by fluorescence microscopy. (c) RAW 264.7 macrophages were transiently transfected with GFP-LC3 and pretreated with PrA/EF (10 µg/mL) or Ctx (5 µg/mL) and KT5720 (5 µM) or vehicle (MeOH) where indicated, 1 h prior to incubation with human IgG opsonised latex beads. Cells were fixed 1 h post-incubation. Extracellular beads were immunostained and not quantified. Quantification of LC3+ beads was performed for at least 100 internalized beads by fluorescence microscopy. (d) Henle-407 cells were pretreated with 6-bnz-cAMP (500 µM), forskolin (10 µM) or IBMX (1000 µM) 1 h prior to invasion with RFP-Sal. Cells were maintained in the indicated conditions during invasion and were fixed at 2 or 10 h post-infection. Bacterial number per infected cell was quantified by fluorescence microscopy and categorized into five categories (1-5, 6-10, 11-15, 16-20 and >20) for at least 100 cells. The percentage of infected cells for each category was quantified and compared with control conditions. Data represents the mean ± SEM for three independent experiments, * indicates $P < 0.05$ and ** indicates $P < 0.01$. 
that upon treatment with these drugs, significantly more cells contained 20 or greater bacteria per cell, further indicating a role for cAMP in the inhibition of antimicrobial autophagy.

cAMP-elevating toxins can inhibit other forms of autophagy

In order to test whether cAMP-elevating toxins were able to inhibit other forms of autophagy, we tested a role for Edtx in rapamycin-induced autophagy (Klionsky et al., 2008). Mammalian target of rapamycin (mTOR) is a well established inhibitor of autophagy (Noda and Ohsumi, 1998). Rapamycin treatment alleviates mTOR’s inhibitory effects resulting in induction of autophagy. HeLa cells were transfected with GFP-LC3 and treated with PrA and EF alone or together in the presence or absence of rapamycin. We observed similar increases in the levels of APs per cell during rapamycin treatment in the presence of growth medium, PrA or EF alone. However, co-treatment with Edtx caused a significant decrease in the number of these structures per cell, indicative of an inhibitory role in non-selective rapamycin-induced autophagy (Figure 4-6A, B).

Autophagy contributes to homeostasis of cellular organelles, including peroxisomes. Kim et al. have previously shown that labelling peroxisomes with monoubiquitin is sufficient to target them for degradation by autophagy (Kim et al., 2008). Using this model, we tested whether treatment with Edtx or Ctx would have an effect on autophagy-mediated clearance of peroxisomes. HeLa cells were transiently transfected with PMP34 C-terminally tagged with GFP-UBko (G76V). PMP34 is a multispansing peroxisomal integral membrane protein that colocalizes with peroxisomes, and GFP-UBko (G76V) is a ubiquitin mutant (that cannot be conjugated to other proteins at Glycine 76) fused to the C-terminus of GFP (Jones et al., 2001; Kim et al., 2008). Expression of this construct has been previously shown to target endogenous peroxisomes to APs for degradation (Kim et al., 2008).

To determine whether these toxins were able to inhibit autophagy-mediated degradation of peroxisomes, PMP34-GFP-UBko (G76V) transfected cells were treated with Edtx or Ctx for 24 h and the percentage of cells with less than 50 peroxisomes was
Figure 4-6 – cAMP-elevating toxins can inhibit rapamycin-induced AP generation.

(a,b) HeLa cells were transiently transfected with GFP-LC3 and treated with PrA (5 µg/mL), EF (10 µg/mL) or rapamycin (25 µg/mL) where indicated. Cells were fixed 2h post-treatment. (a) Representative images are shown for each condition, arrowheads indicate autophagosomes, size bar represents 10 µm. (b) Quantification of the number of round and hollow LC3⁺ APs/cell for 100 cells was performed by fluorescence microscopy. Data represents the mean ± SEM for three independent experiments.
quantified. HeLa cells containing less than 50 peroxisomes were considered to have a reduced number of peroxisomes as they typically contain between 150-300 peroxisomes depending on cell cycle. Under control conditions the majority of cells contained less than 50 peroxisomes indicative of normal peroxisome clearance due to the expression of ubiquitinated peroxisomal proteins (Figure 4-7A, B). Treatment with the general autophagy inhibitor 3-methyladenine (3-MA) resulted in a significant decrease in the number of cells with less than 50 peroxisomes, indicating an impairment in autophagy-mediated clearance. We observed that treatment with Edtx or Ctx also resulted in a significant decrease in the number of cells with less than 50 peroxisomes. We tested the effect of pharmacological agents on this process and observed that the majority of control cells treated with vehicle contained less than 50 peroxisomes. However, treatment with 6-bnz-cAMP, forskolin or IBMX resulted in a significant decrease in the number of cells with less than 50 peroxisomes, indicative of defective pexophagy (Figure 4-7A, C). Therefore, the bacterial toxins Edtx and Ctx can inhibit specific autophagy of organelles, indicating the potential for a general inhibitory role in this process. Furthermore, similar results observed with cAMP/PKA modulating drugs indicate a general role for cAMP in autophagy inhibition.

**DISCUSSION**

It is now appreciated that autophagy plays an integral role in mediation of immune functions in response to microbial infection. While certain bacterial pathogens have been observed to impair autophagic degradation by evading targeting or through inhibition of AP maturation, to date, no bacterial factor has been identified that is capable of inhibiting the initiation of autophagy in host cells. Here we show that two different cAMP-elevating toxins: Edtx from *B. anthracis* and Ctx from *V. cholera* are capable of inhibiting multiple types of autophagy in mammalian cells including antibacterial, FcγR-mediated, rapamycin-induced and clearance of peroxisomes. These toxins had limited cytotoxicity under the conditions used, indicating their actions are specific to the signal transduction cascades that they modulate in host cells (Figure 4-8A, B, C). We further show that manipulation of the
**Figure 4-7 – cAMP-elevating toxins can inhibit other forms of autophagy.**

(a,b,c) HeLa cells were transiently transfected with PMP34-GFP-UBko (G76V) and treated with growth medium (GM), PrA (5 µg/mL), EF (10 µg/mL), Ctx (1 µg/mL), 6-bnz-cAMP (1000 µM), forskolin (20 µM) or IBMX (1000 µM) where indicated. Cells were fixed 24 h post treatment. (a) Representative fluorescent and bright field images are shown for each condition; inner panels represent a higher magnification of the boxed area, size bar represents 10 µm.. (b,c) The percentage of cells with less than 50 peroxisomes/cell was quantified for at least 200 cells by fluorescence microscopy. Data represents the mean ± SEM for three independent experiments, * indicates $P < 0.01$ and ** indicates $P < 0.001$. 
Figure 4-8 – cAMP-elevating toxin and drug conditions used were not cytotoxic.

(a) RAW 264.7 macrophages were treated with GM, DMSO, Edtx (PrA/EF - 10 mg/mL), Ctx (5 mg/µL), 6-bnz (500 µM), forskolin (forsk, 100 µM), IBMX (10 µM), KT5720 (KT, 5 µM) and SDS (0.5%) for 1 hr. (b) HeLa cells were treated with GM, DMSO, 3-MA (10 µM), Edtx (PrA - 5 mg/mL, EF - 10 mg/mL), Ctx (1 mg/mL), 6-bnz (1000 µM), forskolin (forsk, 20 µM), IBMX (1000 µM) and SDS (0.5%) for 24 hrs. (c) Henle-407 cells were treated with GM, Edtx (PrA - 5 mg/mL, EF - 10 mg/mL), Ctx (1 mg/mL), DMSO, 6-bnz (500 µM), forskolin (forsk, 10 µM), IBMX (1000 µM) and SDS (0.5%) for 11 hr. (a-c) Cytotoxicity was assayed using the Celltiter 96 Aqueous Cell Proliferation assay (Promega, Cat # G5430). Absorbance values (A490 nm) were normalized to control GM or DMSO conditions and are presented as a percentage. Data represents the mean ± range for two independent experiments.
cAMP-PKA pathway with pharmacological agents replicates these results, indicative of a role for cAMP and PKA in the regulation of this process. Intriguingly, both *B. anthracis* and *V. cholera* express other toxins, the metalloprotease lethal factor (LF) and *V. cholera* cytolysin (VCC) respectively, that have been shown to be degraded by autophagy (Gutierrez et al., 2007; Tan et al., 2009). This raises the potential for toxin co-operativity, whereby one toxin (Edtx or Ctx) inhibits autophagy in order to allow for enhanced survival of the other, autophagy-targeted toxin (i.e. LF or VCC). Autophagy has been previously shown to play a role in antigen presentation (Dengjel et al., 2005; Schmid et al., 2007). Therefore, autophagy inhibition by cAMP-elevating toxins can also potentially negatively impact on this process facilitating secondary or tertiary infection. Our findings suggest that autophagy inhibition by cAMP-elevating toxins also provides an immediate benefit to the pathogen: by blocking xenophagy, internalized bacteria avoid degradation in the lysosome. It is noteworthy that autophagy is required for the generation of antimicrobial peptides in lysosomes (Alonso et al., 2007; Ponpuak et al., 2010). Therefore, autophagy inhibition by cAMP-elevating toxins may also decrease the bactericidal nature of lysosomes.

The mechanism by which cAMP inhibits autophagy in mammalian cells remains unclear. The effect we have observed on autophagy in the presence of cAMP-elevating toxins and pharmacological agents could be due to a defect in LC3 processing or a defect in cargo targeting and formation of autophagosomes. To test the former possibility, we examined LC3 processing under conditions where autophagic flux is blocked with bafilomycin (Figure 4-9A). We find that cAMP elevation by treatment with Edtx or 6-bnz-cAMP did not affect formation of LC3-II, which accumulated after 2 h treatment with bafilomycin. Formation of LC3-II was also not affected by cAMP elevation when cells were grown in starvation medium (EBSS) and autophagic flux was blocked by bafilomycin (Figure 4-9B). Our data indicate that LC3 processing is not affected, suggesting that cAMP elevating toxins disrupt a downstream step in autophagosome formation.

Previous work in yeast has identified PKA specific phosphorylation sites on two critical autophagy proteins: Atg1 and Atg13 (Budovskaya et al., 2005; Stephan et al., 2009; Yorimitsu et al., 2007). PKA-mediated phosphorylation of these factors resulted in an
impairment in Atg1 and Atg13 localization to the phagophore assembly site, resulting in a defect in autophagy. The possibility therefore exists that phospho-regulation of mammalian homologs of Atg1 and Atg13 also occurs in mammalian cells. It is also possible that cAMP/PKA inhibit normal phagophore development resulting in a defect in cargo targeting and subsequent autophagosome formation. In addition to Atg1 and Atg13 phosphorylation, recent work done in mammalian cells has also identified a putative PKA-phosphorylation site on LC3, with LC3 phosphorylation being inhibitory to this process (Cherra, III et al., 2010). Further studies are required to determine how the cAMP/PKA pathway impacts on autophagy regulation and its consequences for immunity.

cAMP has been observed to act as a general modulator of immune function. Recently, a study of *M. tuberculosis* characterized the existence of a bacterial adenylate cyclase that allowed for increased virulence and bacterial survival (Agarwal et al., 2009). Our work suggests that this effect could be a result of autophagy inhibition, though this remains to be tested. Another group studying *S. aureus* has found that this pathogen is able to synthesize adenosine and that this is required for escape from phagocytic clearance (Thammavongsa et al., 2009). Extracellular adenosine is able to increase intracellular cAMP levels through activation of cell surface adenosine receptors (Feoktistov and Biaggioni, 1997). Whether *S. aureus* escapes killing through inhibition of autophagy by cAMP-elevation after internalization is an intriguing possibility. Another bacterial pathogen, *Listeria monocytogenes* has been shown to be targeted by autophagy (Birmingham et al., 2007; Birmingham et al., 2008; Meyer-Morse et al., 2010; Yano et al., 2008; Yoshikawa et al., 2009). A small molecule screen performed by Lieberman *et al.* identified pimozide as an inhibitor of *L. monocytogenes* infection (Lieberman and Higgins, 2009). Pimozide can block cAMP production and is a calmodulin antagonist (Weissman and Johnson, 1976; Wilson et al., 1984). It is possible that pimozide increases autophagy through inhibition of cAMP production thereby negatively affecting bacterial survival.

Bacteria utilize many strategies to elevate cAMP in host cells (Agarwal et al., 2009; Cassel and Pfeuffer, 1978; Feoktistov and Biaggioni, 1997; Hewlett et al., 1976; Katada and Ui, 1982; Leppla, 1982; Thammavongsa et al., 2009). Indeed, there exist over 600 putative
Figure 4-9 – Elevated cAMP does not affect LC3 processing.

(a,b) HeLa cells were pretreated for 1 hr with Edtx (PrA - 5 µg/mL, EF - 10 µg/mL) or 6-bnz (500 µM) where indicated. Cells were subsequently treated for 2 hrs with (a) GM, Edtx or 6-bnz ± bafilomycin A1 (100 nM) or (b) EBSS, Edtx or 6-bnz ± bafilomycin A1 (100 nM). Cells were harvested and cell lysates analysed by Western blotting. Antibodies used include those against LC3 and β-tubulin.
bacterial adenylate cyclases (Baldari et al., 2006). Our data suggests that bacteria containing cAMP-elevating toxins may utilize them as a general strategy to inhibit cellular autophagy, as well as other immune functions. In addition to resulting in enhanced bacterial survival and virulence, toxin-mediated autophagy inhibition can potentially impact upon many other cellular processes linked to autophagy. Defects in autophagy have been observed in many human diseases including inflammatory bowel disease, cancer, myopathies and neurodegenerative diseases. Whether a link exists between bacterial cAMP-elevating toxins and these human diseases remains to be determined.
CHAPTER 5
DISCUSSION

THE ROLE OF DIACYLGLYCEROL AND PKC IN AUTOPHAGY

The potential for interplay between lipid second messengers and autophagy is attractive due to the importance of membrane dynamics in this process. While some lipids have been observed that can play a role in regulation of autophagy, they all seem to be upstream and lack any targeting function. One key signaling lipid is PI3P produced by Atg14L and UVRAG complexes containing the Class III PI3K hVps34 and has been shown to play a key role in the regulation of autophagy (Yang and Klionsky, 2010). While required for this process, PI3P does not appear to play a specific targeting role but rather functions upstream of this process to induce autophagy through recruitment of specific autophagy components (Yang and Klionsky, 2010). Interestingly, PI3P has been observed on the inner membrane of autophagosomes in yeast, although the significance of this event remains to be determined (Obara et al., 2008).

As described in Chapter 3 of this thesis, I have identified and characterized a novel signaling role for the lipid second messenger DAG in autophagy. I found that DAG is required for autophagy of S. Typhimurium as well as rapamycin-induced autophagy. DAG was observed on bacteria within damaged SCVs and was generated via the actions of PLD and PAP. This signal was found to colocalize with bacteria independent of a functional autophagy pathway (atg5⁻/⁻ MEFs), indicating that it was upstream of autophagosome formation and not recruited via membrane from the autophagy pathway. The δ isoform of protein kinase C, which is activated by DAG, was found to be required for efficient autophagy of the bacteria. Furthermore, I determined that inhibition of both DAG and ubiquitin-mediated autophagy pathways resulted in additive inhibition of S. Typhimurium autophagy, indicative of two separate and distinct targeting pathways. While I have established a role for DAG and PKC in autophagy, certain questions still exist regarding the mechanism of DAG generation, the interplay between DAG and ubiquitin as well as the
potential role for DAG, other signaling lipids and PKCs in general autophagy. In the subsequent subsections I will discuss these issues.

A) Diacylglycerol and Autophagy

1. Mechanism of Diacylglycerol Generation

One important question regarding DAG and autophagy of S. Typhimurium is the mechanism by which DAG is generated on the SCV and the subcellular localization of PLD and PAP during this process. DAG generation could occur through at least two possible mechanisms. In the first, PAP acts directly on the damaged SCV, converting PA to DAG (Figure 5-1). In this scenario, PLD could be generating PA at a separate and distinct site or could also be acting directly on the SCV similarly to PAP. It is interesting that the two isoforms of PLD are considered to have distinct subcellular locations. PLD1 is generally observed in perinuclear membrane structures such as late endosomes or lysosomes whereas PLD2 is typically observed at the plasma membrane (Vorland et al., 2008). I tested both isoforms of PLD and observed a negative effect on autophagy in the presence of dominant negative constructs to each isoform (Figure 3-6E). How PLD1 and PLD2 acting at different sites play a similar role in this process is unknown and should be the subject of future study. I have performed preliminary live cell imaging experiments looking at the localization of PLD2 during Salmonella invasion and observed PLD2 at the plasma membrane during the course of imaging. Upon addition of S. Typhimurium to the cells and induction of membrane ruffling by the bacteria, I observed a concomitant increase in PLD2 levels at sites of bacterial invasion at the plasma membrane. Upon bacterial internalization, I found that PLD2 remained on the SCV (Figure 5-2).

The recruitment of PLD2 to the plasma membrane during bacterial invasion may not be autophagy-related but its localization to the SCV subsequent to internalization may be. As described previously, during the invasion process a subpopulation of S. Typhimurium can damage the SCV and be targeted by autophagy.
Figure 5-1 – Autophagic targeting of S. Typhimurium.

Invading S. Typhimurium can be targeted to the autophagy pathway by two independent signaling mechanisms. The first requires ubiquitin and the autophagy adaptors p62 and NDP52. The second requires DAG generation and PKCδ function. DAG generation on the SCV may occur through interaction of the SCV with DAG-positive endocytic vesicles (pathway 1) or through direct DAG production on the SCV (pathway 2). SCV, Salmonella-containing vacuole; PA, phosphatidic acid; DAG, diacylglycerol; PAP, phosphatidic acid phosphatase; PKCδ, protein kinase C delta; Ub, ubiquitin. Figure adapted from (Shahnazari et al., 2011).
Figure 5-2 – Phospholipase D2 localization during *S. Typhimurium* invasion.

HeLa cells were transfected with PLD2-GFP, infected with RFP expressing *S. Typhimurium* and imaged over the course of the infection. Images corresponding with the indicated time points p.i. are shown. Arrowheads indicate areas where PLD2 is localizing to an invading bacterium.
The possibility exists that PLD2 dissociates from intact SCVs but is maintained on damaged SCVs allowing for the production of PA. The mechanism by which PLD2 can detect damage to a membranous compartment and maintain its position is unknown. The role of PLD1 in this process is also unclear. Since it is typically found at perinuclear regions and colocalizes with late endosomes, one possibility is that it generates PA at these distinct sites and that it is these PA+ vesicles that fuse by an unknown mechanism with damaged SCVs thereby providing the PA substrate for DAG conversion. In this model, regardless of the mechanism by which PA localizes to the SCV, PAP is somehow recruited and converts the PA to DAG allowing for downstream signalling to PKC\(\delta\).

A second possible pathway involves the recruitment to and fusion of DAG+ vesicles with the damaged SCV (Figure 5-1). In this scenario, PAP would act at a site distinct from the SCV. Using a siRNA strategy I was able to determine that the principal isoform of PAP involved in DAG generation and autophagy of \textit{S. Typhimurium} is PAP2B (Figure 3-6D). Similar to other members of the lipid phosphate phosphatase family, PAP2B contains six transmembrane domains and is typically found on the plasma membrane as well as endosomal compartments (Carman and Han, 2006). Interestingly, the active site of these enzymes faces the outer surface of the plasma membrane or the lumen of internal membranous compartments (Brindley, 2004). It is difficult to explain how PAP would gain access to PA generated on the cytoplasmic surface although lipid or even protein flipping is a possibility.

At the same time, another question involves the mechanism by which DAG+ vesicles are specifically recruited to damaged SCVs. This process could potentially involve secondary regulatory factors although further work needs to be done in order to confirm this. I was able to determine that the DAG probe (PKC\(\delta\)-C1-GFP) localized to perinuclear transferrin and Rab5-positive endosomes. In addition to being potential sources for DAG+ vesicles, these sites could also contain regulatory factors that would help in the directed fusion of these vesicles with the bacterial phagosome.

While both pathways are distinct, I believe that they are not mutually exclusive. In fact, using live cell imaging techniques I have been able to observe both mechanisms of
DAG generation on the SCV. In cells transfected with the DAG probe (PKCδ-C1-GFP), I have observed examples where DAG is generated directly on the SCV (Figure 5-3). I have also observed the recruitment and fusion of DAG+ vesicles with the SCV resulting in a coating of DAG around the entire bacteria (Figure 5-4). The existence of two different mechanisms for DAG generation at the SCV or recruitment to this site could simply be a semi-redundant mechanism that ensures the reliability of this system. Further research is required in order to fully dissect the contribution of each pathway to this process.

2. Diacylglycerol and Other Types of Autophagy

In addition to the roles I have shown for DAG in autophagy of *S. Typhimurium* and rapamycin-induced autophagy recent work by two other groups have also found a role for DAG in autophagy. Using a pharmacological inhibitor of DGK, Takita *et al.* observed an increase in the induction of autophagy in response to serum starvation (Takita *et al.*, 2011). While DGK inhibition results in an increase in cellular DAG it also results in a decrease in cellular PA, it is therefore difficult to determine whether the observed increase of autophagy is due to an increase in DAG or decrease in PA. It is also difficult to determine whether DAG is playing a targeting role if any. Surprisingly, Takita *et al.* determined that the induction of autophagy in response to DGK inhibition occurred in a PKC-independent manner. If DAG is in fact playing a role in this process, the identity of its downstream effector also needs to be determined.

Another group, Gorentla *et al.* observed the negative regulation of mTOR by DGKs (Gorentla *et al.*, 2011). The group observed that DAG was required for mTOR activation in T-cells and that DGK inhibits this activation by reducing cellular DAG levels. As described previously, when active, mTOR is an inhibitor of autophagy and while the group did not specifically look at autophagy, this work points to a potential role for DAG as a negative regulator of autophagy. It is of note that the DAG species that this group was studying is generated by PLC (Gorentla *et al.*, 2011). The fatty acid composition of DAG generated by PLC (poly-unsaturated acyl chains) differs from DAG generated by PLD/PAP (mono-
Figure 5-3 – Diacylglycerol generation on the *Salmonella*-containing vacuole.

HeLa cells were transfected with PKCδ-C1-GFP, infected with wild-type *S. Typhimurium* stained with Alexa Fluor 647 carboxylic acid, succinimidyl ester (blue) and imaged over the course of the infection.
Figure 5-4 – Diacylglycerol positive vesicle fusion with the *Salmonella*-containing vacuole.

HeLa cells were transfected with PKCδ-C1-GFP, infected with wild-type *S. Typhimurium* stained with Alexa Fluor 647 carboxylic acid, succinimidyl ester (blue) and imaged over the course of the infection. Arrowheads indicate bacterium fusing with DAG⁺ vesicles.
unsaturated and saturated acyl chains) (Carrasco and Merida, 2007). Certain DAG effectors have been found to have an affinity for particular DAG species (Hodgkin et al., 1998). It is therefore possible that different effects on autophagy are mediated by different DAG species. Whether DAG plays a targeting role for the autophagy of other bacterial species or even other types of autophagy remains to be determined.

B) PKC and Autophagy

In addition to the role I have found for PKCδ in autophagy of S. Typhimurium and rapamycin-induced autophagy, work done in yeast by our collaborators also shows a role for Pkc1 in starvation-induced autophagy (Figure 3-10). The fact that PKC functions in both mammalian and yeast autophagy is indicative of an evolutionarily conserved role for this kinase. To this end, multiple mammalian PKC isoforms have been found to have roles both as promoters of autophagy as well as inhibitors of this process. The majority of PKC isoforms found to play a role in autophagy are DAG-responsive members of the novel PKC family although a direct role for DAG has not been tested in all cases.

Using general pharmacological activators and inhibitors of PKC, Zhang et al. have shown that PKC promotes oridonin-induced autophagy (Zhang et al., 2009). Oridonin is a naturally occurring diterpenoid compound that can induce an autophagic response (Cui et al., 2006). Using similar techniques, Jiang et al. were able to determine that activation of PKC actually inhibited autophagy as determined through LC3-I to LC3-II conversion in response to starvation or rapamycin (Jiang et al., 2010). I have previously described a pro-autophagy function for PKCδ in hypoxia-induced autophagy and PKC0 in autophagy in response to ER stress (Chen et al., 2008; Sakaki et al., 2008). An opposing view has been proposed by Akar et al. who have found that inhibition of PKC0 can result in an increase in autophagy in pancreatic cancer cells (Akar et al., 2007). Due to these contradictory results, further work needs to be done in order to fully characterize the role of this kinase family in autophagy. It is possible that the pro or anti-autophagy function of PKC isoforms is dependent on the specific type of autophagy as well as cell type. It is also possible that due
to their multiple signalling functions, they could in fact be operating both as activators and inhibitors of this process depending on the step of the autophagy pathway at which they function. At the same time other PKC isoforms DAG-responsive or not may also be found to function in autophagy.

If PKC isoforms are in fact determined to be activators of autophagy, another important question that remains to be answered is how they activate this pathway. Two proposed models exist. The first mechanism proposed by Chen et al. has been confirmed for a general autophagic response to acute hypoxia. They determined that a signalling pathway that requires JNK-mediated phosphorylation of Bcl-2 allowed for the dissociation of Beclin 1 from Bcl-2 and subsequent induction of autophagy (Chen et al., 2008). While they propose that JNK activation and phosphorylation of Bcl-2 in this model is PKCδ-dependent the model does not explain how PKCδ itself is activated in response to the stressor.

The second potential mechanism by which PKCs induce autophagy is through ROS production. Work done by our lab has determined a signalling role for ROS in autophagy of S. Typhimurium as well as LC3 localization to phagosomes (Huang et al., 2009). Huang et al. have shown that ROS production through the NADPH oxidase complex is necessary for this process. Formation of the NADPH oxidase complex and ROS production requires the spatiotemporal assembly of multiple components at the phagosome. It is known that translocation of one component, p47phox from the cytosol to the phagosome requires PKC-mediated phosphorylation (Bertram and Ley, 2011). We therefore propose that PKCδ functions to activate autophagy by promoting the assembly of NADPH oxidase and the subsequent generation of ROS. While this model is an attractive one, the mechanism by which ROS are able to signal and activate autophagy remains to be determined. In Chapter 3 of this thesis, I have tested both models of PKC-mediated induction of autophagy and found that both contribute (Figure 3-11). It is therefore possible that there does not exist one single mechanism by which autophagy is activated by PKCs but rather it could occur through a combination of both pathways that again depend upon cell as well as autophagy...
Determination of the exact mechanism(s) of autophagy induction should also be the subject of future investigation.

C) Other Signalling Lipids and Autophagy

While PI3P is the major lipid species that plays a regulatory role in autophagy, other signalling lipid species have also been found to play a role in autophagy. Sphingolipids including ceramide and sphingosine 1-phosphate (S1P) as well as PA and phosphatidylinositol 4-phosphate (PI4P) have also been recently shown to play a stimulatory role in autophagy. While principally associated with the induction of cell death (Hannun and Obeid, 2008) ceramide has also been shown to stimulate autophagy in a manner similar to starvation-induced autophagy (Scarlatti et al., 2004). The group of Patrice Codogno has been able to identify at least three different pathways by which ceramides are able to stimulate autophagy. Scarlatti et al. showed that ceramide is able to stimulate autophagy through the inhibition of Akt/protein kinase B signalling and as a result mTOR activation (Scarlatti et al., 2004). They were also able to show that increased intracellular ceramide levels also increases the expression of Beclin 1 (component of the autophagy-stimulating Class III PI3K complex). In addition to these two pathways, Pattingre et al. were recently able to show that ceramide can also activate JNK1 (Pattingre et al., 2009). Beclin 1 can be found in a complex with the anti-apoptotic protein Bcl-2, this complex excludes Beclin 1 binding to the Class III PI3K complex thereby disrupting autophagy (Pattingre et al., 2005). Pattingre et al. determined that JNK1 activation in response to ceramide phosphorylates Bcl-2 promoting the dissociation of Beclin 1 thereby allowing for induction of autophagy. Interestingly, JNK mediated activation of autophagy also correlates with our own results showing a role for JNK in autophagy of S. Typhimurium (Figure 3-11A, B). In our model, PKCδ activated by DAG activates JNK promoting Beclin 1 dissociation. Another sphingolipid found to play a role in stimulation of autophagy is S1P. Similar to ceramide, S1P has been shown to inhibit mTOR activity although unlike ceramide, this occurs in an Akt/protein kinase B-independent manner (Lavieu et al., 2006). Additionally, unlike
ceramide, S1P is thought to act as a mediator of starvation-induced autophagy and does not induce autophagy on its own (Lavieu et al., 2007).

In addition to being a metabolite for DAG production, PA has itself been recently shown to function in the autophagy pathway. Work by Dall’Armi et al. has identified an important role for PLD1 in autophagy (Dall’Armi et al., 2010). They initially observed a partial translocation of PLD1 from a perinuclear region to the outer membrane of APs during starvation conditions and determined that it plays a role in maturation of these compartments. They further found that inhibition of PLD1 function resulted in decreased LC3-I to LC3-II conversion and altered the size and number of APs indicating a role for the PLD1 product PA in this process. While the group observed PA directly on APs it was not present at a high level. Dall’Armi et al. speculate that the low observable level of PA could be due to a transient role for this lipid or that it is converted to DAG. How exactly PLD1-produced PA functions in this pathway remains to be fully characterized.

1. Phosphatidylinositol 4-phosphate and Autophagy

The final signalling lipid that I would like to discuss is PI4P. Only one study has found a role for PI4P in autophagy in yeast while no work has been done in mammalian systems. PI4P was found to play a role in micropexophagy in the yeast Pichia pastoris (Yamashita et al., 2006). Yeast micropexophagy involves the invagination of the yeast vacuole (lysosome) and formation of two arms that extend to surround the peroxisome resulting in the internalization of this organelle (Farre and Subramani, 2004). A structure called the micropexophagy-specific membrane apparatus (MIPA) has been shown to be required for the nucleation of autophagy components and efficient internalization of peroxisomes by this pathway (Mukaiyama et al., 2004). Yamashita et al. observed that PI4P production was required for the formation of the MIPA and that it functioned to recruit an autophagy protein (PpAtg26) that promoted the maturation and formation of this structure. Interestingly, in my initial lipid screen looking at the colocalization of signalling lipids with autophagy targeted and non-autophagy targeted S. Typhimurium I also tested PI4P and
Figure 5-5 – Phosphatidylinositol 4-phosphate localization with S. Typhimurium and rapamycin-induced autophagosomes.

(a,b) HeLa cells were co-transfected with RFP-LC3 and either 2FYVE-GFP (PI(3)P probe), PLCδ-PH-GFP (PI(4,5)P2 probe), GFP-PH-AKT (PI(3,5)P2 and PI(3,4,5)P3 probe), PKCδ-C1-GFP (DAG probe) or FAPP1-PH-GFP (PI(4)P probe). (a) Cells were infected with wild-type S. Typhimurium, fixed at 1 h p.i. and immunostained for S. Typhimurium. The percentage of RFP-LC3⁺ or RFP-LC3⁻ bacteria colocalizing with the lipid probes in was determined by fluorescence microscopy. (b) Cells were treated with rapamycin (25 µg/mL) and fixed 2 h post-treatment. The percentage of RFP-LC3⁺ autophagosomes colocalizing with each lipid probe was determined by fluorescence microscopy.
observed significantly more colocalization of PI4P with LC3\(^+\) bacteria when compared to LC3\(^-\) bacteria (Figure 5-5A). I additionally found significant colocalization of PI4P with rapamycin-induced APs (Figure 5-5B). While no mammalian ortholog of Atg26 exists, further characterization of a role for PI4P in mammalian autophagy and the identity of its downstream effector should be the subject of future study. The possibility exists that PI4P plays a targeting role similar to that of DAG.

**D) Two Pathways Mediate Bacterial Targeting by Autophagy**

Our lab has previously determined that autophagy of *S. Typhimurium* occurs in a ubiquitin-dependent manner but that only 50% of autophagy-targeted bacteria colocalize with ubiquitinated proteins (Birmingham et al., 2006; Zheng et al., 2009). With the identification of DAG as another targeting mechanism, the possibility exists that DAG and ubiquitin are functioning in a cooperative manner to target *Salmonella* for degradation by autophagy. To this end, I simultaneously inhibited both pathways using siRNA and pharmacological agents and observed additive inhibitory effects on autophagy of the bacteria. While this is indicative of two independent and separate pathways (Figure 5-1), I cannot discount the possibility that there is still cooperation between the two pathways, especially as I did observe a small population of bacteria that were positive for both DAG and ubiquitin. Furthermore, there are a number of technical limitations in the methods we used, such as detection levels of the probes and antibodies that warrant caution in concluding that the two pathways are completely independent.

In order to fully dissect the contribution of DAG and ubiquitin to autophagy of *Salmonella* many questions remain to be answered. It is well established that SCV damage is the initial signal that targets this compartment for degradation by autophagy (Birmingham et al., 2006). This membrane damage may expose the bacteria to the cytosol facilitating the ubiquitination of bacterial proteins. At the same time, since the identity or identities of the ubiquitinated protein(s) is unknown the possibility also exists that a host protein associated with the SCV is the target of ubiquitination. Regardless of the identity or identities of the
ubiquitinated protein(s), ubiquitin-mediated autophagy requires ubiquitin-LC3 adaptors capable of bridging both signaling pathways. By binding to ubiquitin moieties and interacting with LC3+ membrane, these adaptors such as p62 or NDP52 allow for the specific targeting of these cargos.

On the other hand, due to its very nature, DAG colocalization with the SCV is most likely as part of the SCV membrane itself. This sets up an attractive system where DAG is generated in response to bacteria-induced membrane damage allowing for targeting of the SCV by autophagy, resulting in degradation of this compartment and associated bacteria. DAG localization to L. monocytogenes-containing vacuoles has also been observed in response to membrane damage caused by Listeriolysin O (Shaughnessy et al., 2007). It is therefore possible that DAG generation occurs in response to general membrane damage regardless of cause, facilitating an autophagic response. This could be a damaged organelle or a damaged bacteria-containing phagosome. At the same time, if the ubiquitinated protein(s) involved in autophagy of S. Typhimurium are determined to be bacterial in origin then this second system would function as a clearance mechanism for direct bacterial targeting by autophagy independent of membrane damage. Both pathways function independently of each other and respond to different stimuli but can occur at the same time when both signals are present on the same SCV with the DAG pathway acting in response to membrane damage and ubiquitin mediating the targeting of the bacteria itself. While this hypothesis is a possibility, additional research is required in order to fully dissect these pathways. Given the importance of autophagy targeting in response to bacterial invasion I believe that this will be an exciting area of study.
THE ROLE OF cAMP AND PKA IN AUTOPHAGY

Microbial targeting by the autophagy pathway is now appreciated to be an integral component of the immune response. Multiple viral, Gram-positive and Gram-negative bacterial species have been identified as targets of this process (Deretic and Levine, 2009). In response to this defence, certain microbes have developed strategies that allow them to evade autophagic degradation. Some species can avoid autophagy-mediated targeting (Ogawa et al., 2005; Yoshikawa et al., 2009) while others can inhibit AP maturation (Birmingham et al., 2008). These strategies affect autophagy locally, and do not appear to affect the induction of autophagy elsewhere in the cell. Viral species have been identified that can suppress initiation of autophagy (Deretic and Levine, 2009). One factor expressed by herpes simples virus type 1 is ICP34.5 that can inhibit autophagy at an early stage through interaction with the key autophagy component Beclin 1 (Orvedahl et al., 2007). To date, no bacterial factor has been identified that is capable of inhibiting the initiation of autophagy.

In Chapter 4 of this thesis I have identified a mechanism by which bacteria can inhibit autophagy on a cellular level by increasing intracellular cAMP. Utilizing two different bacterial toxins Edtx and Ctx from *B. anthracis* and *V. cholera* respectively, I observed inhibition of multiple types of autophagy through direct cAMP production or activation of host cAMP machinery. Additionally, using pharmacological agents that mimic increases in cAMP concentration, I have shown a direct inhibitory role for cAMP and its effector, PKA in this process. The effect that I observed on distinct types of autophagy is indicative of a general inhibition of cellular autophagy. Having identified a novel bacterial defence strategy, further work still remains to be done in order to determine whether other bacteria also take advantage of this system as well as identifying the precise mechanism by which autophagy is inhibited through this process.
A) Other Bacterial cAMP-elevating Factors

In addition to *B. anthracis* and *V. cholera*, many other bacterial species have been found to express factors capable of increasing intracellular cAMP levels either directly through AC activity or indirectly through activation of host AC machinery. In this section, I will describe the known bacterial species that express cAMP-elevating toxins (Table 5-1). *Bordetella pertussis* (*B. pertussis*) is a Gram-negative pathogen that is the causative agent of the respiratory illness known as pertussis (or whooping cough). Pertussis is a contagious and acute illness that typically manifests itself with severe coughing and vomiting (Mattoo and Cherry, 2005). Two bacterial factors that are important for *B. pertussis* virulence are Pertussis toxin (Ptx) and CyaA (Carbonetti, 2010). Ptx is a 115 kDa secreted protein comprised of an A subunit and five B subunits, the pentameric B subunits can bind to cell surface receptors and are internalized along with the associating A subunit (de Gouw et al., 2010). The A subunit is similar to Ctx in that it functions as an ADP-ribosyltransferase but differs in target as it transfers ADP-ribose to Gαi, inhibiting Gαi-mediated inhibition of host ACs allowing for increased cAMP production (Mattoo and Cherry, 2005). The second *B. pertussis* virulence factor is CyaA which is a secreted 200 kDa that is internalized through interaction with cell surface receptors on phagocytes (Carbonetti, 2010). Once within the cytosol it is activated and functions as an AC increasing intracellular cAMP levels.

Enterotoxigenic *Escherichia coli* (ETEC) is a common cause of traveller’s diarrhea and results in between 280-400 million cases annually, mostly within the developing world (Taxt et al., 2010). Diarrheal symptoms can range from mild to severe and can result in dehydration and exhaustion (Fleckenstein et al., 2010b). One of ETEC’s principal toxins is heat-labile enterotoxin (HLtx) which shares approximately 80% sequence homology with Ctx (Salmond et al., 2002). Similar to Ctx, HLtx is composed of a single A subunit and five B subunits. The B subunit pentamer binds to GM1 gangliosides and is internalized with the associated A subunit (Fleckenstein et al., 2010b). The internalized A subunit acts as an ADP-ribosyltransferase, activating Gαs subunits that subsequently activate host ACs increasing cAMP levels (Salmond et al., 2002).
### Table 5-1 – Bacterial pathogens that express cAMP-elevating toxins.

<table>
<thead>
<tr>
<th>Bacterial Species</th>
<th>Gram-staining</th>
<th>Toxin Type</th>
<th>Toxin Name</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Bacillus anthracis</em></td>
<td>positive</td>
<td>AC toxin</td>
<td>Edema factor</td>
</tr>
<tr>
<td><em>Bordetella pertussis</em></td>
<td>negative</td>
<td>AC toxin</td>
<td>CyaA</td>
</tr>
<tr>
<td></td>
<td></td>
<td>ADP-ribosyltransferase</td>
<td>Pertussis toxin</td>
</tr>
<tr>
<td>Enterotoxigenic <em>Escherichia coli</em> (ETEC)</td>
<td>negative</td>
<td>ADP-ribosyltransferase</td>
<td>Heat-labile toxin</td>
</tr>
<tr>
<td><em>Mycobacterium tuberculosis</em></td>
<td>N/A</td>
<td>AC toxin</td>
<td>Rv0386</td>
</tr>
<tr>
<td><em>Pseudomonas aeruginosa</em></td>
<td>negative</td>
<td>AC toxin</td>
<td>ExoY</td>
</tr>
<tr>
<td><em>Vibrio cholera</em></td>
<td>negative</td>
<td>ADP-ribosyltransferase</td>
<td>Cholera toxin</td>
</tr>
<tr>
<td><em>Yersinia pestis</em></td>
<td>negative</td>
<td>AC toxin</td>
<td>CyaA</td>
</tr>
</tbody>
</table>
*Mycobacterium tuberculosis* (*M. tuberculosis*) is an intracellular bacterial species that can infect macrophages and dendritic cells. Once internalized it prevents the maturation of the phagosome it occupies through an as yet undetermined mechanism (Rohde et al., 2007b). *M. tuberculosis* has 17 genes that encode putative ACs (Shenoy et al., 2004). Recently, one of these genes, Rv0386, a cell wall associated AC has been found to be required for *M. tuberculosis* virulence (Agarwal et al., 2009). Agarwal *et al.* found that infection of mice with a strain of *M. tuberculosis* lacking Rv0386 resulted in reduced PKA responses and virulence. They also determined that bacterial-derived cAMP within the phagosome can cross the phagosomal membrane and increase within the host cell cytosol. It is of note that *M. tuberculosis* has been shown to be targeted by autophagy when pharmacologically or physiologically induced (Gutierrez et al., 2004). It would be interesting to test whether Rv0386-deficient *M. tuberculosis* can be targeted by autophagy without prior stimulation.

*Pseudomonas aeruginosa* (*P. aeruginosa*) is a Gram-negative opportunistic pathogen that can cause a wide range of acute and chronic infections including those of the respiratory tract, urinary tract, wounds and bloodstream (Hauser, 2009). This species is unique in that it has a relatively large genome encoding six different classes of secretion systems (Bleves et al., 2010). One effector secreted through its TTSS is the adenylate cyclase, ExoY. ExoY differs from Edtx and CyaA because it is delivered to host cells through direct injection via *P. aeruginosa*’s TTSS (Yahr et al., 1998). Therefore, whereas Edtx and CyaA can act at sites distal to their source, ExoY injection requires close contact between bacteria and target cell. If the toxin is acting to inhibit autophagy, this could provide the bacteria with localized autophagy inhibition rather than a systemic and wide ranging effect.

Another bacterial species that is known to express a cAMP-elevating toxin is *Yersinia pestis* (*Y. pestis*). *Y. pestis* is the highly pathogenic causative agent of the plague. Among its numerous virulence factors exists CyaA an extracellular AC that has not been very well characterized but results suggest that it can contribute to bacterial pathogenesis (Ahuja et al., 2004). In addition to the above described pathogens, a search of the NCBI protein database for “bacterial ACs’ returns over 1000 putative proteins from a diverse range of
bacteria. The possibility therefore exists that known and putative cAMP-elevating toxins produced by various bacterial species can also inhibit autophagy in a manner similar to that which I have shown for Edtx and Ctx. This could be a general strategy utilized by these bacteria to promote their pathogenesis and enhance virulence.

B) Potential Benefits of Bacteria-mediated Autophagy Inhibition

Bacteria-mediated PKA activation through cAMP production has been implicated in the negative regulation of certain immune-related functions including phagocytosis and ROS production (Serezani et al., 2008). Autophagy itself also plays a large role in many innate and immune-related cellular responses including direct targeting of intracellular bacteria. Due to its role in multiple cellular processes, bacterial-mediated inhibition of autophagy can have multiple benefits for the bacteria. In this section, I will discuss potential outcomes for \textit{B. anthracis} and \textit{V. cholerae} upon inhibition of autophagy.

\textit{V. cholerae} infection generally occurs within the gastrointestinal tract, similarly, \textit{B. anthracis} infection can also occur within this system upon ingestion. Recent work has attributed a role for autophagy in the function of ileal epithelial Paneth cells. Paneth cells are specialized intestinal epithelial cells at the base of intestinal crypts that function to secrete antimicrobial factors into the intestinal lumen (Cadwell et al., 2009). Secretion of these factors occurs through the exocytosis of secretory granules containing defensive compounds such as secretory phospholipase 2A, lysozyme, LPS-binding protein and metallothionein that can target a broad range of bacteria (Ouellette, 2010). Cadwell et al. have determined that reduced levels of the autophagy protein Atg16L lead to cellular deficiencies in Paneth cells (Cadwell et al., 2008). This included morphological abnormalities, abnormal mitochondria, reduced number of granules as well as deficiencies in the granule exocytosis pathway. Since it was possible that the role of Atg16L in Paneth cells is independent of autophagy, the group also tested mice deficient for other autophagy components including Atg5 and Atg7 and observed similar results indicating a role for autophagy in Paneth cell function (Cadwell et al., 2009). Based on these results the
possibility exists that the inhibition of autophagy by bacteria using cAMP-elevating toxins in the gastrointestinal tract could provide the bacteria with a safer niche promoting bacterial replication and growth.

*B. anthracis* infection can occur via inhalation of inactive spores (Tournier et al., 2009). Once within the lung epithelium, these spores are phagocytosed by macrophages and transferred to lymph nodes where the spores germinate, escape and replicate prior to entering the bloodstream (Tournier et al., 2009). In the lymph node as well as the bloodstream, Edtx secreted by *B. anthracis* could affect multiple immune cells including B and T lymphocytes. Autophagy has been shown by multiple groups to play a role in the development, maintenance and function of these cells. Pua et al. observed an impairment in the survival and proliferation of mature T cells in autophagy-deficient mice (Atg5 KO) (Pua et al., 2007). Additionally, the same group also showed a role for autophagy in T cell homeostasis (Pua et al., 2009). Furthermore, autophagy has been found to play a crucial role in the development of pre-B cells within the bone marrow (Miller et al., 2008).

Autophagy has been previously described as being involved in the delivery of endogenous antigens for MHC class II presentation (Deretic and Levine, 2009). More recently, Lee et al. provide evidence for a role for autophagy in the presentation of exogenous antigens (Lee et al., 2010). The group examined Atg5 KO dendritic cells and found an impairment in the processing and presentation of phagocytosed TLR agonist antigens for MHC class II presentation to T cells resulting in a defect in T cell priming. Based on the role of autophagy in lymphocyte maturation and survival as well as antigen presentation, the inhibition of this process by the bacteria would have multiple beneficial effects. By affecting core components of the adaptive immune system, the bacteria could potentially prevent the development of antibodies and immune memory facilitating secondary or tertiary infections once initially cleared.

In addition to specific immune-related functions, as described previously autophagy also plays a role in many other pathways. The inhibition of cellular autophagy by *V. cholera* and *B. anthracis* could also simply result in general cytotoxicity. By inhibiting autophagy, these bacteria can affect general cellular homeostasis and metabolism resulting in the
accumulation of protein and protein aggregates as well as damaged or unneeded organelles. During nutrient deprived conditions, the inhibition of autophagy could also act to exacerbate an already stressful condition thereby adversely affecting cells.

A final, intriguing possible outcome of autophagy inhibition for these bacteria is the concept of toxin cooperativity. It is of note that in addition to their cAMP-elevating toxins, both *V. cholera* and *B. anthracis* secrete other toxins that have been found to be targeted by autophagy. *V. cholera* cytolysin (VCC) is a secreted 79 kDa pore-forming toxin that forms heptameric β-barrel transmembrane pores in cell membranes resulting in vacuolization and cell lysis (Lohner et al., 2009). Gutierrez *et al.* observed an autophagic response in cells treated with VCC (Gutierrez *et al.*, 2007). The group found that the generation of VCC-induced vacuoles was autophagy dependent and that they colocalized with LC3. Interestingly, they also determined that autophagy plays a protective role in response to VCC. Using autophagy-deficient MEFs and autophagy inhibitors, they found that cells lacking autophagy were much more susceptible to treatment with VCC. Cell viability and survival dropped drastically in autophagy-deficient cells compared to WT cells indicative of a pro-survival role for autophagy in response to VCC and vacuolation (Gutierrez *et al.*, 2007). More recently, Tan *et al.* found that the zinc metalloprotease Ltx from *B. anthracis* can also induce an autophagic response in treated cells (Tan *et al.*, 2009). Cells treated with Ltx had an increase in LC3-I to LC3-II conversion as well as an increase in LC3⁺ puncta, indicative of an induction of autophagy. Similar to the results observed with VCC, upon inhibition of autophagy the group observed accelerated cell death during co-treatment of cells with Ltx and the autophagy inhibitor 3-MA indicating a protective role for autophagy in response to intoxication (Tan *et al.*, 2009). Interestingly, in an unrelated study testing the susceptibility of macrophages to Ltx, the researchers found that the autophagy inducer rapamycin played a protective role promoting cell survival while autophagy inhibitors such LY294002 and wortmannin enhanced cell death (Kim *et al.*, 2003). While the authors were not directly testing a role for autophagy in this process, the results are indicative of a protective role for autophagy against LF.
In the case of both *V. cholera* and *B. anthracis*, activation of autophagy occurs in response to toxin treatment and could be acting as a mechanism to clear the toxins from the cell. Therefore, based on these and my own results, the possibility exists that in *V. cholera* strains that secrete both Ctx and VCC and *B. anthracis*, the toxin pairs function cooperatively. Inhibition of autophagy by Ctx or Edtx allows for enhanced intoxication and intracellular longevity for VCC and Ltx respectively. Both Ctx and Edtx have been clearly shown to have their own function within cells in the promotion of virulence including the inhibition of autophagy (Ahuja et al., 2004; De Haan and Hirst, 2004). The aspect of toxin cooperativity may not be their principal mode of action but rather simply a secondary aspect to their primary function. The possibility exists that this strategy also occurs in other bacterial species that express cAMP-elevating toxins. Interestingly, among bacterial species known to containing cAMP-elevating toxins, certain species such as *B. pertussis, P. aeruginosa,* and *Y. pestis* also secrete other toxins into host cells. *B. pertussis* expresses a TTSS, the sole effector protein of this system that has been identified is the 69 kDa protein, BteA. BetA contains an N-terminal lipid-raft targeting domain and can induce rapid cell death (French et al., 2009). In addition to its AC-toxin, *P. aeruginosa* also secretes a phospholipase A$_2$ (ExoU) and two proteins with GTPase-activating domain and ADP-ribosyltransferase activity (ExoS and ExoT) (Bleves et al., 2010). *Y. pestis* can also secrete numerous toxins into host cells via its TTSS including a GTPase-activating protein (YopE), a protein tyrosine phosphatase (YopH) and a toxin that disrupts actin filaments (YopT) (Huang et al., 2006). Whether these toxins are also targeted by autophagy in a manner similar to that shown for VCC and Ltx remains to be determined and should be the subject of further work.

**C) cAMP/PKA and Autophagy**

One important area of future study that remains is the characterization of the exact mechanism by which the cAMP/PKA signalling pathway inhibits autophagy. While the majority of research done in this field has occurred in yeast, the pathways and mechanisms...
identified may be translated into the mammalian system. In this section, I will describe in more detail the existing pathways for the negative regulation of autophagy via cAMP/PKA and propose alternate possibilities.

The first direct connection between PKA signalling and autophagy was determined by a proteomics approach in *S. cerevisiae*, screening for potential PKA phosphorylation sites (Budovskaya et al., 2005). Among the putative phosphorylation targets of PKA identified in their screen was the yeast ortholog of the serine/threonine kinase Ulk (Atg1). Atg1 in yeast, much like Ulk in mammalian cells functions at an early stage of autophagy initiation and is required for the induction of this process. Interestingly, PKA phosphorylation of Atg1 had no direct effect on its activity but instead resulted in mislocalization of this protein (Budovskaya et al., 2005). When nutrient levels are normal, Atg1 is present within the cytosol but under nutrient starvation conditions it relocalizes to the PAS and induces autophagy. Budovskaya *et al.* found that Atg1 mutants lacking PKA phosphorylation sites were constitutively present at the PAS regardless of nutrient levels. Constitutive activation of PKA through AC activation maintained Atg1 in the cytosol and blocked relocalization to the PAS during nutrient deprivation. This is suggestive of a regulatory role for this kinase where PKA-mediated phosphorylation of Atg1 maintains Atg1 within the cytosol whereas dephosphorylation at the PKA sites permits relocalization to the PAS and subsequent induction of autophagy. In their study, Budovskaya *et al.* also found that two other autophagy components, Atg13 and Atg18 contain potential PKA phosphorylation sites.

More recent work done by the same group in the yeast system has been able to further dissect this pathway. Stephan *et al.* found that similar to Atg1, the Atg1-interacting protein Atg13 is also phosphorylated by PKA and that this controls Atg13 localization to the PAS (Stephan et al., 2009). As described in Chapter 1, both Ulk1 (mammalian Atg1) and mAtg13 (mammalian Atg13) are phosphorylated by mTOR and these phosphorylation events control the interaction between the two as well as their localization to the PAS. Stephan *et al.* were able to show that phosphorylation events catalyzed by TOR and PKA occur at different residues, independently of each other and that inhibition of both pathways results in an enhanced induction of autophagy (Stephan et al., 2010). While TOR
controls the interaction between Atg1/Atg13 and PAS localization, PKA only controls the
PAS localization of these proteins. Why two pathways exist for the regulation of Atg1/Atg13
remains to be fully characterized. It is possible that PKA-mediated phosphorylation of Ulk
and mAtg13 also occurs in mammalian systems and plays a similar role in the regulation of
autophagy. Whether this involves the regulation of the localization of these proteins is a
possibility but is complicated by the fact that the mammalian PAS has yet to be identified.
Interestingly, proteomic work done on mouse Ulk1 by Dorsey et al. has identified a putative
PKA phosphorylation site although the downstream effects of this event have yet to be
determined (Dorsey et al., 2009).

In addition to phosphorylation of Ulk and mAtg13, another potential target for PKA
in mammals is LC3. A novel study by Cherra et al. has proposed that LC3 is phosphorylated
in a PKA-dependent manner (Cherra, III et al., 2010). This is the first time that LC3 has been
suggested to be phosphorylated and proposes an interesting regulatory mechanism. Using
mass spectrometry techniques, Cherra et al. identified a PKA phosphorylation site at the
serine 12 residue of LC3. Activation of host ACs with forskolin resulted in an increase in LC3
phosphorylation while induction of autophagy corresponded with dephosphorylation of LC3
(Cherra, III et al., 2010). A phospho-mimetic mutant of LC3 also resulted in impaired
recruitment to APs in response to rapamycin treatment. Phospho-LC3 comigrated with LC3-
I suggesting that it is the nonlipidated form of this protein that is phosphorylated and that
this occurs prior to conversion to LC3-II. While this preliminary study reveals a hitherto
unknown mechanism of autophagy regulation, the impact of LC3 phosphorylation on
autophagy induction and the mechanism by which this occurs remains to be determined.

Regulation of autophagy by cAMP/PKA could also occur independently of its kinase
activity. As described previously, PKA is a holoenzyme comprised of two regulatory and two
catalytic subunits. Mavrakis et al. observed that regulatory subunit 1-α (R1α) of PKA
associated with late Rab7-positive endosomes as well as LC3+ APs and that there is a
decrease in the average number of APs per cell in R1α KO MEFs (Mavrakis et al., 2006). The
group also found that R1α interacts with mTOR and that mTOR activity is increased in the
absence of this subunit. It is possible that the observed effect on autophagy is simply due to
the activation of the catalytic subunits of PKA in the absence of R1α but the authors propose that R1α directly interacts with mTOR and this interaction inhibits its activity promoting autophagy. Finally, cAMP/PKA are also regulated by PDEs (convert cAMP to AMP) and AKAPs (compartmentalize PKA signalling) whether these factors also play a role in autophagy regulation remains to be determined. Having described potential mechanisms by which cAMP/PKA can inhibit autophagy, further investigation is required in order to conclusively characterize this pathway. Given the potential therapeutic and health benefits of cAMP modulation in response to bacterial infection this will be an exciting area of future research.

CONCLUSION

The work presented in this thesis has identified roles for two second messengers in the targeting of autophagy as well as the regulation of this process. Diacylglycerol was found to localize to Salmonella-containing vacuoles independently of ubiquitin and prior to targeting by the autophagy pathway. The identification of a requirement for the diacylglycerol effector PKCδ downstream of this lipid in antibacterial and rapamycin-induced autophagy is suggestive of a general role for this kinase in the induction of this process. The discovery of diacylglycerol as a novel signal in the identification of targets for autophagy should be the subject of future study in the context of other cargos. Additionally, for the first time a novel bacterial strategy of autophagy inhibition using cAMP-elevating toxins has been described. cAMP elevation through two distinct mechanisms by Bacillus anthracis and Vibrio cholerae was found to result in PKA activation and concomitant autophagy inhibition. Multiple bacteria contain similar toxins and the possibility exists that autophagy inhibition by this mechanism is a general tactic utilized by these microbes. The mechanism of target identification and regulation of autophagy will continue to be areas of future research and will provide multiple therapeutic benefits given the important role autophagy can play in both the protection from and promotion of human disease.
Reference List


