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FLORIDA INTERNATIONAL UNIVERSITY

Miami, Florida

MECHANISM OF SUPEROXIDE MEDIATED REGULATION OF PARTICLE UPTAKE AND EXOCYTOSIS BY A GPI-ANCHORED SUPEROXIDE DISMUTASE C IN *DICTYOSTELIUM*

A thesis submitted in partial fulfillment of the

requirements for the degree of

MASTER OF SCIENCE

in

BIOLOGY

by

Maria Pulido

2014

To: Interim Dean Michael R. Heithaus
College of Arts and Sciences

This thesis, written by Maria Pulido, and entitled Mechanism of Superoxide Mediated Regulation of Particle Uptake and Exocytosis by a GPI-anchored Superoxide Dismutase C in *Dictyostelium*, having been approved in respect to style and intellectual content, is referred to you for judgment.

We have read this thesis and recommend that it be approved.

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Date of Defense: June 27, 2014

The thesis of Maria Pulido is approved.

Interim Dean Michael R. Heithaus
College of Arts and Sciences

Dean Lakshmi N. Reddi
University Graduate School

Florida International University, 2014

DEDICATION

I dedicate this thesis to my best friend and mother Judith Navarro. Your unconditional love and support has been the cornerstone of my life. Also to my father Carlos Pulido and the rest of my family, thank you for the support you have given me and for making sure I achieve my goals. Finally I would also like to thank all the teachers and professors I have ever had, especially my major advisor Dr. Lou W. Kim. You have all shaped me into who I am today and I could not be more grateful.

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ABSTRACT OF THE THESIS

MECHANISM OF SUPEROXIDE MEDIATED REGULATION OF PARTICLE UPTAKE AND EXOCYTOSIS BY A GPI-ANCHORED SUPEROXIDE DISMUTASE C IN *DICTYOSTELIUM*

by

Maria Pulido

Florida International University, 2014

Miami, Florida

Professor Lou W. Kim, Major Professor

Dictyostelium discoideum is a simple model organism that can be used to study endocytic pathways such as phagocytosis and macropinocytosis because of its homology to cells of the mammalian innate immune system, namely macrophages and neutrophils. Consequently, *Dictyostelium* can also be used to study the process of exocytosis. In our laboratory, we generated *Dictyostelium* cells lacking superoxide dismutase SodC. Our data suggest that cells that lack SodC are defective in macropinocytosis and exocytosis when compared to wild type cells.

In this study I describe a regulatory mechanism of macropinocytosis by SodC via regulation of RasG, which in turn controls PI3K activation and thus macropinocytosis. Our results show that proper metabolism of superoxide is critical for efficient particle uptake, for the proper trafficking of internalized particles, and a timely exocytosis of fluid uptake in *Dictyostelium* cells.

TABLE OF CONTENTS

CHAPTER PAGE

Chapter 1: Introduction ... 1
1.1 Summary ... 1
Chapter 2: Literature review ... 2
2.1. *sodC*$^{-}$ cells provide a unique opportunity to study the role of ROS in phagocytic cells ... 2
2.2 Endocytosis ... 4
2.2.1 Phagocytosis ... 4
2.2.2 Macropinocytosis ... 6
2.3 Inhibitory role of the PI3K inhibitor LY294002 in the process of macropinocytosis ... 8
2.4 Exocytosis ... 9
Chapter 3: Methodology ... 11
3.1 *Dictyostelium* development and growth ... 11
3.2 Particle uptake assay ... 11
3.3 Quantification of FITC-latex beads internalized ... 12
3.4 Particle uptake assay using a phosphatidylinositol 3-kinase inhibitor ... 12
3.5 Exocytosis assay ... 13
3.6 Statistical analysis ... 13
Chapter 4: Results ... 14
4.1 General aspects of particle uptake in wild type and *sodC*$^{-}$ cells. ... 14
4.2 Quantitative analysis of particle uptake in wild type and *sodC*$^{-}$ cells. ... 15
4.3 *sodC*$^{-}$ cells are defective in macropinocytosis ... 16
4.4 *sodC*$^{-}$ cells are defective in exocytosis ... 17
4.5 Figures ... 18
Chapter 5: Discussion ... 26
5.1.1 Relevance to mammalian cells ... 29
5.2 Future perspective ... 30
References ... 32

LIST OF FIGURES

Figure 1: Regulation of phagocytosis and macropinocytosis occurs by two different biochemical pathways 18

Figure 2: Particle uptake in wild type *Dictyostelium discoideum* cells 19

Figure 3: Particle uptake in *sodC*$^{-}$ *Dictyostelium discoideum* cells 20

Figure 4: Particle uptake by wild type and *sodC*$^{-}$ *Dictyostelium* cells 21

Figure 5: Particle uptake by wild type and *sodC*$^{-}$ *Dictyostelium* cells after addition of the PI3K inhibitor LY294002 22

Figure 6: Comparison of particle uptake in wild type and *sodC*$^{-}$ *Dictyostelium* cells before and after the addition of the PI3K inhibitor LY294002 23

Figure 7: Particle uptake in wild type and *sodC-* *Dictyostelium* cells after addition of the PI3K inhibitor LY294002 24

Figure 8: Exocytosis of FITC-dextran in wild type and *sodC*$^{-}$ *Dictyostelium* cells 25

ABBREVIATION AND ACRONYMS

Abbreviation	Meaning
ABP120	Actin binding protein-120
CLC3	Chloride channel-3
DAG	Diacylglycerol
DAip1	*Dictyostelium* actin-interacting protein 1
EC-SOD	Extracellular superoxide dismutase
F-actin	Filamentous actinl
FITC	Fluorescein isothiocyanate
GFP	Green Fluorescent Protein
GTPase	Guanosine trisphosphate phosphohydrolase
IP$_3$	Inositol-1,4,5-Triphosphate
IRF-3	Interferon regulatory factor 3
IRF-7	Interferon regulatory factor 3
LmpA	
NADPH	Nicotinamide adenine dinucleotide phosphate
NF$_k$B	nuclear facto- kappaB
Nox	NADPH oxidase
PI3K	Phosphoinositide 3 kinase
PI(3)P	Phosphatidylinositol 3-phosphate
PI(3.4)P$_2$	Phosphatidylinositol 3,4-bisphosphate
PI(3,4,5)P$_3$	Phosphatidylinositol 3,4,5-trisphosphate
PIK1	Phosphatidylinositol kinase 1
PIK2	Phosphatidylinositol kinase 2
PIP3	Phosphatidylinositol 3,4,5-trisphosphate

PKB……………………………………………………...……...……… Protein kinase B

PLC……………………………………..…...………………..… Phospholipase C

Rap1………………………………………………………… Ras-related protein RAP-1A

RasGEFB……………………………………………..… Ras guanine exchange factors B

ROS……………………………………………………....……Reactive oxygen species

SodC…………………………………………………….……Superoxide dismutase c

Chapter 1: Introduction

1.1 Summary

The organism *Dictyostelium discoideum* is a unicellular eukaryote that reproduces by binary fission and undergoes multicellular development in the absence of food (Williams *et al*., 2006). *Dictyostelium discoideum* is a "professional" phagocyte and this trait makes it an excellent model to study the molecular mechanisms of chemotaxis and phagocytosis (Maniak, 2002; Annesley and Fisher, 2009). Cells of the human immune system such as neutrophils and macrophages destroy invading microbial pathogens by phagocytosis, which is preceded by chemotaxis. It is not surprising then that some of the proteins involved in chemotactic motility are also involved in phagocytosis, although these proteins are controlled by different upstream receptors and Ras proteins depending on the process (Bozzaro *et al*., 2008).

The processes of chemotaxis and phagocytosis are not fully understood, not only in their molecular nature but also mechanistically. Furthermore, other processes of particle and fluid uptake such as macropinocytosis and pinocytosis, respectively, are yet starting to be fully comprehended.

In our laboratory we were able to generate *Dictyostelium discoideum* cells lacking a glycosylphosphatidylinositol (GPI)-anchored superoxide dismutase (SodC). Cells that lack Superoxide Dismutase C, experience oxidative stress as a result of modestly increased intracellular superoxide level (~18% higher compared to wild type), high basal

RasG activity and defects in chemoattractant sensing, cell polarization and motility (Veeranki *et al*., 2008).

To identify which and how the processes of particle uptake and exocytosis are affected in *Dictyostelium discoideum* cells under oxidative stress, we performed a series of assays in which we fed 1 μm FITC-latex beads and FITC-dextran to wild type and *sodC⁻* *Dictyostelium discoideum* cells in order to quantify particle uptake and exocytosis, respectively.

Chapter 2: Literature review

2.1. *sodC⁻* cells provide a unique opportunity to study the role of ROS in phagocytic cells.

The release of reactive oxygen species (ROS) occurs normally as a result of oxygen metabolism in the cells. Conversely, the excessive accumulation of reactive oxygen species (ROS) in the cells can prove to be detrimental. The well-studied harmful effects caused by the excessive accumulation of reactive oxygen species (ROS) include: damage to the DNA, lipid peroxidation, oxidation of amino acids and the inactivation of specific enzymes (Brooker, 2011).

One of the functions of ROS that demands further understanding is its role in defense against pathogens in mammalian hosts. A study showed that increased levels of ROS facilitated the signaling to activate interferon regulatory factors three and seven (IRF-3 and IRF-7), and nuclear facto- kappaB (NF_kB) that result in an antiviral state (West *et al.,* 2011). A more recent study also showed that the production of ROS could

be responsible for the secretions of interferon type three to control infection by the influenza A virus (Kim *et al*., 2013).

One of the main reactive oxygen species produced in the cells is superoxide. Normally, cells can defend against superoxide by the family of enzymes of superoxide dismutases. Superoxide dismutases convert superoxide into hydrogen peroxide, which eventually gets converted into oxygen and water. Although the properties of superoxide have been studied in vitro, understanding its role in particle uptake *in vivo* has proven to be a difficult task because superoxide has a high reactivity and a short half-life. In our laboratory we have previously generated *Dictyostelium discoideum* cells lacking one of the superoxide dismutases, SodC, in order to understand the role of superoxide in biological processes such as chemotaxis and particle uptake. SodC is a glycosylphosphatidylinositol (GPI)-anchored enzyme that is located in the outer leaflet of the plasma membrane and is involved in the regulation of intracellular level of superoxide in *Dictyostelium* cells (Veeranki *et al*., 2008).

Dictyostelium discoideum cells behave like cells of the mammalian innate immune system, namely neutrophils and macrophages, phagocytizing bacteria several fold more than neutrophils (Bozzaro *et al*. 2008). *Dictyostelium discoideum* cells lacking *SodC* exhibit high RasG activity, which was shown to be critical for proper phagocytosis (Chen and Katz, 2000). We thus investigated if *sodC*$^-$ cells are defective in particle uptake and other related processes such as macropinocytosis and exocytosis.

2.2 Endocytosis

The term endocytosis refers to all types of plasma membrane internalization, ranging from the formation of small invaginations, 100-200nm in diameter, to the formation of large membranous vesicles several micrometers in diameter. Among the category of large membranous vesicles are phagososmes and macropinososmes, formed during phagocytosis and macropinocytosis, respectively (Bohdanowicz and Grinstein, 2013). The main events of endocytosis in wild type *Dictyostelium* cells are: the formation of an endosome that remains stable in the cell for approximately 1 minute before it starts to lose its coat (consisting of cytoskeletal proteins, including F-actin, and proteins involved in signal transduction) (Maniak *et al.,* 1995; Hacker *et al.,* 1997; Rupper *et al.,* 2001; Peracino *et al.,* 1998; Konzok *et al.,* 1999; Insall *et al.,* 2001; Maniak, 2003); the uncoating of the endosome; acidification of the uncoated endosome via fusion with other vesicles that contain the vacuolar H^+ATPase (Clarke *et al.,* 2002); maturation of the acidified endosome from which two processes occur, early recycling and fragmentation (Maniak, 2003); and finally, after subsequent events of the endocytic pathway in wild type *Dictyostelium*, exocytosis of indigestible particles and fluid (Maniak, 2003).

2.2.1 Phagocytosis

Phagocytosis is a receptor-mediated process that occurs when particles bigger than 0.5 µm in diameter are recognized by receptors on the surface of professional phagocytes, such as macrophages and neutrophils (Bohdanowicz and Grinstein, 2013).

Phagocytosis involves a number of stages that include recognition and attachment of the cell surface receptors by the binding particles, followed by activation of a signaling

pathway that leads to the engulfment of the particles, and vesicle trafficking that leads to the formation of phago-lysosomes from phagosomes via a series of fusion and fission reactions (Bohdanowicz and Grinstein, 2013).

In *Dictyostelium discoideum* cells two classes of receptors have been recognized to be responsible for the binding of particles at the cell surface. One of these receptors binds to the terminal glucose residues in bacteria, while the other class of receptors, the "nonspecific" receptor, is responsible for the binding of particles such as latex beads by hydrophobic interactions (Cardelli, 2001).

There are at least three membrane proteins in *Dictyostelium* that are responsible for the adhesion of particles to the cell receptors. These proteins are Phg1 (Cornillion *et al.*, 2000), SadA (Fey *et al.,* 2002), and SibA (Cornillion *et al*., 2006). Among these three, SibA has a cytoplasmic domain that binds to a complex between talin and myosin VII; which are important in the adhesion and engulfment of particles in phagocytosis (Cardelli, 2001; Cosson and Soldatti, 2008).

The binding of particles to the cell receptors causes a signaling pathway that causes F-actin to accumulate below the forming phagocytic cup (Maniak *et al.,* 1995; Cardelli, 2001). In addition to F-actin, ABP120, an actin-crosslinking protein, and coronin also accumulate below the forming phagocytic cup and contribute to its formation and stability (Bretschneider *et al*., 2009). The pathway that causes F-actin to be spatially and temporally controlled starts with the binding of particles to the cell receptors, which activates phospholipase C (PLC) through a possible interaction with the β-subunit of the heterotrimeric G protein. Diacylglycerol, a product of PLC, could

possibly activate Rap1 and/or RasS while IP_3, a second product of PLC, increases cytosolic calcium levels. Both, the activation of Rap1 and/or RasS and the increase of cytososlic calcium level, aid in the regulation of phagocytosis. Furthermore, members of the Rho family such as RacC possibly activate Scar, a protein involved in the polymerization of actin filaments. Additionally, Rab GTPases such as RabB and Rab7 might deliver internal membrane to the newly formed phagocytic cup (Cardelli, 2001).

The formation of a phagocytic cup is followed by a series of maturation steps that eventually lead to the degradation of the ingested particles. During the steps of the maturation process, some proteins are selectively excluded while others are added to the phagosomes. The proteins lining each phagosome give its identity. Once the phagosome starts to fuse with lysososmes, particles ingested are degraded. For example, lysosomal hydrolases such as Cathepsin G and elastase are involved in the killing of *S. aureus* and *C. albicans* (Reeves *et al*., 2002; Cosson and Soldati, 2008); whereas β-hexosaminidase is involved in the killing of mycobacteria (Koo *et al*., 2008; Cosson and Soldati, 2008). Another killing mechanism that has been suggested but has been difficult to study in *Dictyostelium* cells involves the production of reactive oxygen species (ROS) by the NADPH-oxidase. Deficiencies in the ability of NADPH-oxidase to produce ROS lead to inefficient intracellular bacterial killing (Cosson and Soldati, 2008).

2.2.2 Macropinocytosis

Macropinocytosis, like phagocytosis, is an actin-dependent but clathrin independent endocytic process. Macropinocytosis differs from phagocytosis in that the macropinosomes are heterogeneous in size, ranging from 0.2 to 5 µm. Furthermore,

macropinocytosis is responsible for the uptake of non-specific fluid, solutes, and particles (Mercer and Helenius, 2012). The process of macropinocytosis occurs in several stages that include particle recognition and binding, followed by the activation of an intracellular signaling pathway that leads to F-actin remodeling and plasma membrane protrusions, formation and closure of a vacuole and lastly, macropinosome trafficking (Mercer and Helenius, 2012).

Particle recognition and binding initiate a signaling pathway that triggers a transient change in cell behavior that leads to the actin-dependent formation of membrane protrusions to engulf the attached particles. Depending on the cell type, conditions, and type of receptors involved, the macropinocytic protrusions can take different forms. Among the different forms that macropinocytic protrusions can take are lamellopodial ruffles, circular ruffles, filopodial protrusions, and blebs (Mercer and Helenius, 2012).

Approximately one minute after macropinosome fission from the plasma membrane is completed, the cytoskeletal coat dissociates from the macropinosome and the vacuolar H^+ATPase acidifies its lumen (Maniak, 2001).

The formation of late endosomes from early endosomes during macropinocytosis is marked by the effector molecules dynamin and vacuolin, which operate in fission of recycling vesicles and target exocytosis, respectively (Maniak, 2001).

Macropinocytosis and phagocytosis share common protein components such as RasS (Chubb *et al*., 2000), coronin (Maniak *et al*., 1995; Cardelli 2001), RasGEFB (Wilkins *et al*., 2000), Rab7 (Cardelli, 2001), Scar (Seastone *et al*., 2001), myosin I’s, RabD, and DAip1. However, different biochemical pathways regulate macropinocytosis

and phagocytosis (Cardelli, 2001). For example, the overexpression of RacC or Rap1 in wild type cells and the loss of profiling impair macropinocytosis but stimulate phagocytosis (Seastone *et al*., 1998; Seastone *et al.,* 1999; Temesvari *et al*., 2000; Cardelli, 2001). Additionally, deletion of the genes encoding PKB, LmpA, and the genes encoding the P110-like PI 3-kinases PIK1 and PIK2 reduces the rate of macropinocytosis but does not affect phagocytosis (Temesvari *et al.,* 2000; Cardelli 2001). A model representing the common and distinct proteins involved in the processes of phagocytosis and macropinocytosis is depicted in Figure 1.

2.3 Inhibitory role of the PI3K inhibitor LY294002 in the process of macropinocytosis

Phosphatidylinositol-3-kinase (PI3K) pertains to a family of enzymes whose multiple functions include intracellular trafficking, motility, differentiation, and proliferation. There are three classes in which the family of phosphoinositol-3-kinase can be identified on the basis of their structure, regulation, and lipid substrate specificity. Tyrosine kinase receptors and G-protein coupled receptors activate Class I of the phosphoinositol-3-kinase. Class I PI3Ks are responsible for the production of Phosphatidylinositol 3-phosphate (PI(3)P), Phosphatidylinositol (3,4)-bisphosphate ($PI(3.4)P_2$), and Phosphatidylinositol (3,4,5)-trisphosphate ($PI(3,4,5)P_3$).

In wild type *Dictyostelium* cells, PIP3 patches develop perpendicular to the forming macropinosomes and remain static during the early stages of the process (Veltman *et al*., 2014). *Dictyostelium* cells that lack all PI 3-kinases have no PIP3 patches (Hoeller and Kay, 2007). Thus PI 3-kinase inhibitors such as LY294002, inhibit the

process of macropinocytosis (Veltman *et al.,* 2014). Earlier studies showed that deletion of the genes encoding the P110-like PI 3-kinases (Class I PI 3-kinases) in *Dictyostelium*, PIK1 and PIK2, greatly reduced macropinocytosis but did not affect phagocytosis (Temesvari *et al*., 2000).

2.4 Exocytosis

Exocytosis is the process by which a cell guides the contents of vesicles out of the membrane and into the extracellular space. In *Dictyostelium* cells there are three steps that define the process of exocytosis and these include vesicle trafficking, vesicle docking, and vesicle fusion.

Dictyostelium discoideum cells take in fluid and solutes from the extracellular environment. During the late stages of vesicle trafficking, undigested material accumulates in the late endosomes and is exocytosed (Maniak, 2003). Right before exocytosis, the WASP and SCAR homologue (WASH) derived F-actin, binds and removes the V-ATPase either directly or as part of a larger protein assembly (Carnell *et al*., 2011) The V-ATPase consists of a membrane channel (V_0) and a cytoplasmic ATP-hydrolyzing proton pump (Jefferies *et al*., 2008) that is responsible for the acidification of the late endosome; thus by removing the V-ATPase the late endosome gets neutralized.

During normal exocytosis, after the V-ATPase has been removed, actin assembles at several points on the late endosome membrane and exocytosis follows (Clarke *et al*., 2010). A study showed that fusion of late endosomes with the plasma membrane created the transient formation of a p-80-rich domain at the cell surface and that the

aforementioned fusion never occurred in sites of the plasma membrane that were undergoing phagocytosis or macropinocytosis, therefore fusion of whole late endocytic compartments is not responsible for membrane delivery to phagocytic or macropinocytic cups or to the cell front (Charette and Cosson, 2006).

Chapter 3: Methodology

3.1 *Dictyostelium* development and growth

The JH10 and *sodC*$^-$ cells were grown using D3T medium (14.3g/l Bacto peptone #3, 7.15g/l yeast extract, 15.4g/l glucose, 0.48g/l KH2PO4, 0.525g/l Na2HPO4). In addition to D3T medium, JH10 cells were grown with thymidine (0.5mg/ml) and *sodC*$^-$ cells were also grown in thymidine (0.5mg/ml) and Blasticidin (5µg/ml). Both types of cells were grown in shaking cultures at 19°C at 150rpm or in culture flasks at 19°C for about 24 hours until they were in log phase. Cell number was monitored using a hemocytometer. Cell number for every experiment performed was between 2x10^6 cells/ml to 2.4x10^6cells/ml.

3.2 Particle uptake assay

A total of three million cells *of Dictyostelium discoideum* growing in the log phase were added to four separate 15 ml centrifuge tubes each. Each 15ml centrifuge tube was spun down at 2000 rpm for 5 min at 4°C. After spinning, cells were resuspended in 1ml of cold D3T media and fluorescent beads were added to each 15 ml centrifuge tube to a final concentration of 50 beads per cell. Cells and beads were shaken at 150 rpm at room temperature for various times, including a start period consisting of 0-2 minutes (0-2 minutes accounts for the amount of time the cells were in contact with the beads before spinning and during spinning), 10min, 20min, and 30min. After shaking, 1x10^6 *Dictyostelium discoideum* cells were added to 10ml of cold DB media and washed 4 times in cold DB. After washing, the final pellet was resuspended in 1ml of DB media at

room temperature and 500µl of the final 1ml suspension were placed into a 4 chamber slide and kept at 4°C for 10 minutes to allow the cells to settle to the surface of the slide.

3.3 Quantification of FITC-latex beads internalized

We used the Leica DM IRB inverted microscope and CoolSNAP-Pro digital camera. We randomly picked 20 *Dictyostelium* cells and took photos at 100x and at different layers of the z-axis to allow for a 3-D view of *Dictyostelium* cells. We took approximately 10 pictures in phase contrast every 2 seconds and adjusted the z-axis before every picture to ensure we detected all FITC-beads. We then took approximately twenty images using the GFP filter every 2 seconds and adjusted the z-axis in every picture. We moved through the z-axis approximately 2 µm between each picture. We then counted the number of internalized beads in each cell in addition to taking pictures at different z-axis.

3.4 Particle uptake assay using a phosphatidylinositol 3-kinase inhibitor

Three million cells *of Dictyostelium discoideum* growing in the log phase were added to four separate 15ml centrifuge tubes each. Each 15ml centrifuge tube was spinned down at 2000rpm for 5min at 4°C. After spinning, cells were resuspended in 1ml of cold D3T media and the phosphatidylinositol 3-kinase inhibitor LY294002 (20 µM) was added to each 15ml centrifuge tube and incubated in shaking for 20 min at room temperature. After the 20 minutes, the assay continued as the particle uptake assay (see above). Quantification of internalized FITC-latex beads was done using the protocol above.

3.5 Exocytosis assay

Total exocytosis of FITC-dextran was measured by following the experimental design by Seastone *et al*., (2001). Three million cells of *Dictyostelium discoideum* growing in the log phase were added to two separate 15ml centrifuge tubes each. Each 15ml centrifuge tube was spinned down at 2000rpm for 5min at 4°C. After spinning, cells were resuspended in 1ml of cold D3T media and FITC-dextran (relative molecular mass (*Mr*) 70,000, Sigma) was added as the fluid marker at a final concentration of 2mg/ml to one of the tubes, the second tube served as control. The two 15ml centrifuge tubes were then covered in aluminum foil and shaken at 150rpm at room temperature for a period of three hours. After three hours in shaking, cells were washed twice in 1 ml of cold D3T media and resuspended in 7 ml of D3T. Then 1 ml aliquots were collected every twenty minutes, spun down at 2000 rpm for 5 min at 4°C and the supernatant was measured for fluorescence using a spectrofluorimeter using the settings described in Seastone *et al*., (2001). Before every 1 ml aliquot was taken cells in the 15 ml centrifuge tubes were resuspended to keep cell volume constant during the experiment. To make sure cell number was constant in every 1 ml aliquot, the cell pellet was used to measure protein concentration following a BCA™ Protein Assay kit.

3.6 Statistical analysis

Statistical analysis of the data was performed using independent t-tests in the SPSS program. The results for the particle uptake assays with and without the addition of the PI3K inhibitor LY294002 were expressed and graphed as mean + standard deviation of the three independently performed sets in both wild type and *sodC*$^{-}$ *Dictyostelium*

discoideum cells, and considered significantly different at $P < 0.05$. The values of percentage fluorescence in the exocytosis assay were calculated measuring the value read in the spectrofluorimeter at 0 min after the three-hour incubation period as 0% and then subsequent values obtained were compared and turned into a percentage with respect to the value read at 0 min in both wild type and *sodC*$^{-}$ *Dictyostelium discoideum* cells. The percentage fluorescence graphed in the exocytosis assay was an average of the percentages at each time point of three different sets in each cell line.

Chapter 4: Results

4.1 General aspects of particle uptake in wild type and *sodC*$^{-}$ cells.

Wild type and *sodC*$^{-}$ cells were incubated with the fluorescent (Fluorescein isothiocyanate (FITC)) latex beads up to 30 minutes as indicated in Figure 2 and Figure 3, and images of randomly chosen twenty cells with internalized latex beads were captured as described in the Methodology. Representative images for each time point were shown in figure 2 and figure 3. The initial time point “Start” realistically represent 1~ 2 minutes because of the unavoidable centrifugation step after mixing the beads with cells. The internalized beads were localized at different depth inside the cell and thus only some of the beads were on the focal plane of each image. The internalized latex beads exhibited noticeable movement, which is reflected in the slightly different positions of beads in the phase contrast and fluorescent images (Fig.2 & Fig.3) that were taken with a time delay (up to a minute).

These internalized beads were generally well dispersed throughout the cytoplasm of wild type cells. In contrast, the latex beads in *sodC*$^-$ cells were fewer in number, and surprisingly most of the beads were entrapped in huge vacuoles of 3~5 micrometers in diameter (Fig. 3).

The intensity of FITC signal inside the cell is reported to be pH dependent (Carnell *et al.,* 2011). Consequently, the intensity of FITC-latex bead decreased as the beads entered acidic compartments of the wild type cells (data not shown).

Wild type cells exhibited around ten-fold increase in particle uptake after ten minutes of feeding time (Figure 4) in JH10 cells when compared to the average of internalized particles during the start period. The start period consisted of 0-2 minutes feeding time. When counting the internalized particles we noticed that the fluorescent beads were dim after ten, twenty, and thirty minutes of feeding time when compared to the start period (data not shown).

4.2 Quantitative analysis of particle uptake in wild type and *sodC*$^-$ cells.

Over the time course of the assay, wild type cells always demonstrated significantly higher levels of particle uptake compared to *sodC*$^-$ cells (Fig.4). The difference in particle uptake between wild type and *sodC*$^-$ cells showed a P value <0.05 at the initial START point and a P value<0.01 at all other time points (Fig.4). Wild type cells internalized 17.5 latex beads per cell during 30 minutes of feeding period, whereas *sodC*$^-$ cells internalized ~ 8 particles per cell post 20 minutes feeding. No significant increase was observed at 30 minutes of feeding, indicating that *sodC*$^-$ cells not only

displayed slower rate of particle uptake, but also exhibited severely reduced particle uptaking capacity.

4.3 *sodC*$^{-}$ cells are defective in macropinocytosis

Given that both phagocytosis and macropinocytosis could mediate particle uptake, it would be possible that *sodC*$^{-}$ cells were defective in either or both of the processes. To determine which is the case, cells were treated for 20 minutes prior to feeding period with PI3K inhibitor, LY294002, which is known to inhibit macropinocytosis, but not phagocytosis of *Dictyostelium* cells (Veltman *et al*., 2014). Wild type cells treated with LY294002 displayed significantly decreased (~ 40%) particle uptake over the time course as shown in Figure 5. In contrast, LY294002 treated *sodC*$^{-}$ cells generally exhibited similar level particle uptake over the assay period (Fig.5 and Fig.7). LY294002 treated *sodC*$^{-}$ cells seemed to have additional defects in uptaking particles at 20 minutes of feeding, but seemingly normal with additional 10 minutes of feeding period. These results suggest that macropinocytosis, but not phagocytosis, is the most significantly compromised particle uptaking mechanism in *sodC*$^{-}$ cells.

The internalized beads after treatment with LY were generally well dispersed throughout the cytoplasm just as described in the particle uptake assay without the LY treatment. Similarly, latex beads in *sodC*$^{-}$ after treatment with LY were still entrapped in huge vacuoles of 3~5 micrometers in diameter (Fig. 7).

4.4 *sodC⁻* cells are defective in exocytosis

Given that macropinocytosis, but not phagocytosis, was the most significantly compromised particle uptaking mechanism in *sodC*$^{-}$ cells (Fig. 5 and Fig.6), and hence macropinocytosis is the main fluid uptake mechanism in *Dictyostelium* cells (Hoeller *et al*., 2013) it would be possible that *sodC*$^{-}$ cells were also defective in the exocytosisof FITC-dextran. To determine if *sodC*$^{-}$ cells were defective in exocytosis, we incubated the cells with fluorescent (Fluorescein isothiocyanate (FITC)) dextran (Mr) 70,000 up to 3 hours and measured exocytosis subsequently every twenty minutes for two hours using a spectrofluorimeter, as described in the Methodology.

Wild type cells exhibited ~90% increase in fluid exocytosis after forty minutes of exocytosis (Fig.8) compared to the initial time pointJH10 cells right after the initial 3 hour incubation period or at 0 minutes after exocytosis (Fig.8). In contrast, *sodC*$^{-}$ cells displayed only ~25% increase in fluid exocytosis after forty minutes of exocytosis compared to the initial time point (Fig.8).

Over the time course of the assay, wild type cells displayed a steady increase in fluid exocytosis that seemed to reach a plateau after 100 minutes of exocytosis (Fig.8). Conversely, *sodC*$^{-}$ cells did not increase much until they reached 80 minutes of exocytosis, after which fluid exocytosis seemed to increase linearly and ~25% faster.

At all time points during the assay, *sodC*$^{-}$ cells displayed a significantly smaller percentage of exocytosed fluid compared to wild type cells.

4.5 Figures

Figure 1

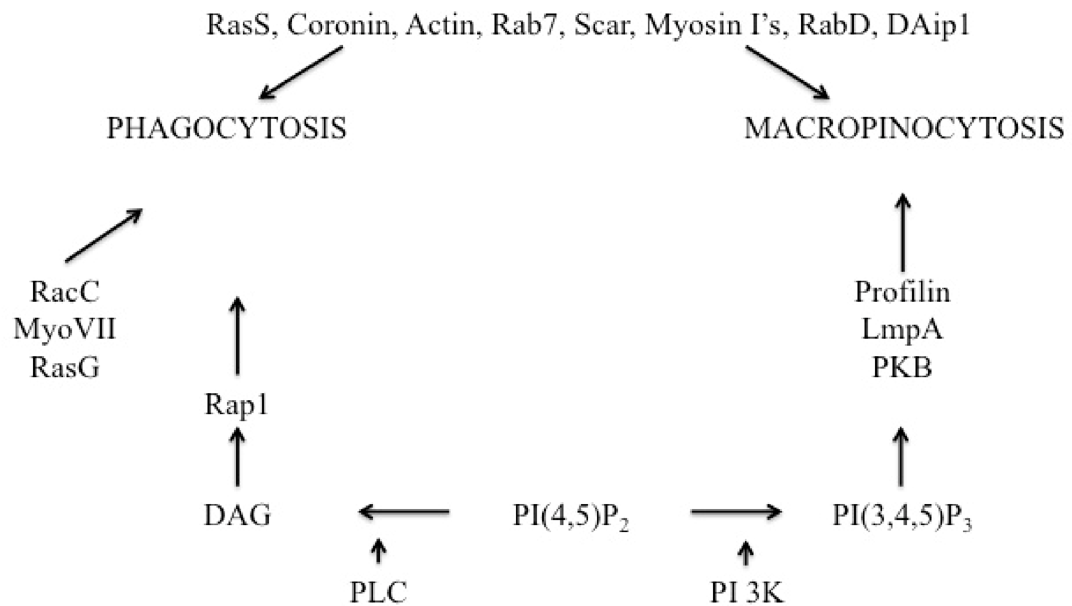

Figure 1: Regulation of phagocytosis and macropinocytosis occurs by two different biochemical pathways

This model shows two possible biochemical pathways by which phagocytosis and macropinocytosis can be regulated. Phagocytosis is initiated when calcium levels increase due to the activation of Rap1. Rap1 is activated by the formation of DAG and IP_3. The formation of DAG and IP_3 is catalyzed by PLC. Macropinocytosis is initiated as a result of the accumulation of PIP3 in the nascent macropinosome. PI 3-kinases catalyze the formation of PIP3. RasS, coronin, actin, Rab7, Scar, myosin, RabD, and DAip1 are proteins involved in phagocytosis and macropinocytosis.

Figure 2

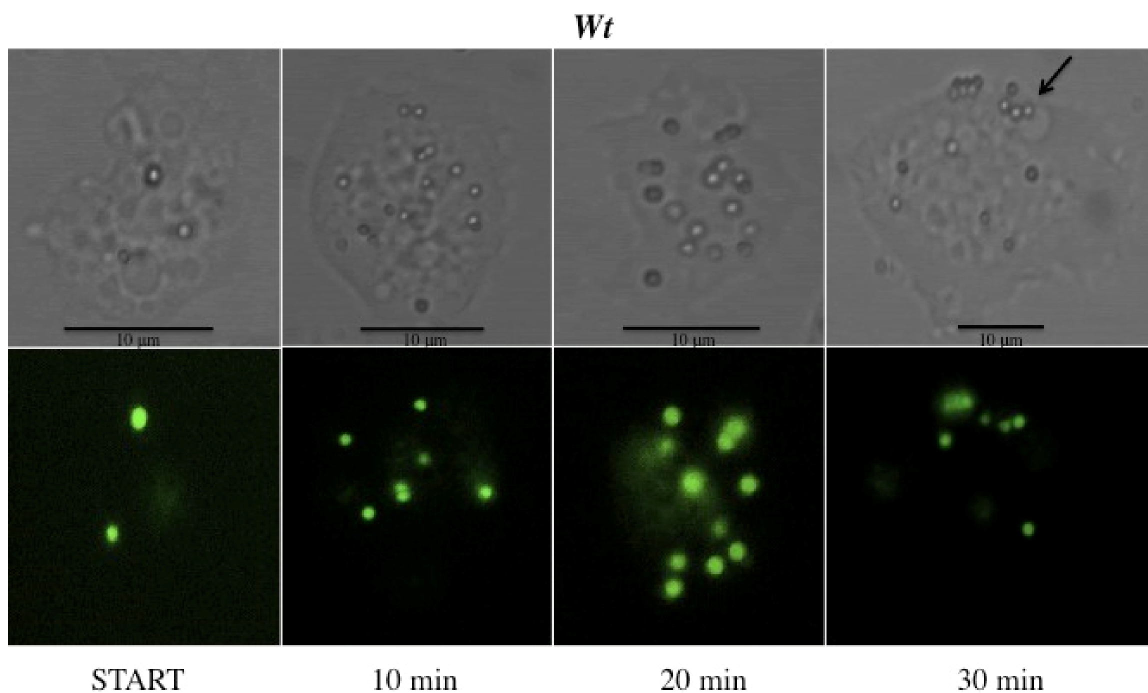

Figure 2: Particle uptake in wild type *Dictyostelium discoideum* cells

This figure shows an increase in particle uptake as feeding time increases. The start period consists of 1 – 2 minutes of feeding FITC-beads to wild type *Dictyostelium* cells (see Methodology). The top pictures are the most representative phase contrast pictures from randomly picked cells during the particle uptake assay. The bottom pictures are the most representative fluorescent pictures that display the greatest number of FITC-beads in a single z-axis point. Pictures were taken with up to a 1 min difference, hence the movement of particles is accounted for the different positions of the beads in the corresponding phase contrast and fluorescent pictures The arrow in the 30min phase contrast picture points at a forming endosome.

Figure 3

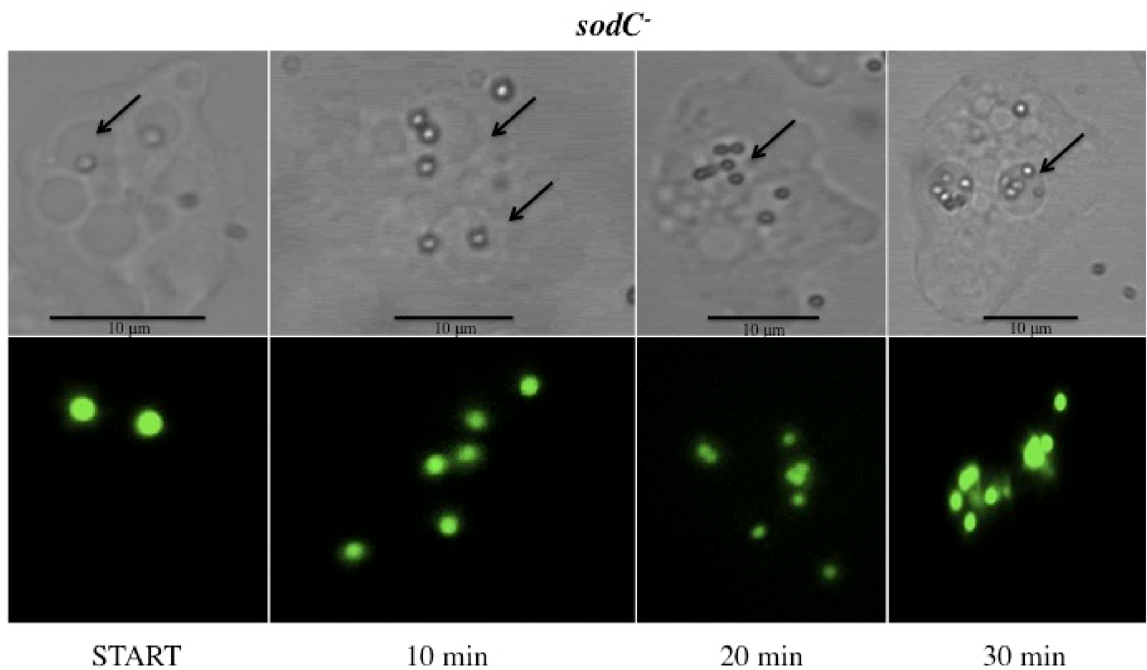

Figure 3: Particle uptake in *sodC*$^-$ *Dictyostelium discoideum* cells

This figure shows an increase in particle uptake as feeding time increases. The start period consists of 1 – 2 minutes of feeding FITC-beads to *sodC*$^-$ *Dictyostelium* cells (see Methodology). The top pictures are the most representative phase contrast pictures from randomly picked cells during the particle uptake assay. The bottom pictures are the most representative fluorescent pictures that display the greatest number of FITC-beads in a single z-axis point. Pictures were taken with up to a 1 min difference, hence the movement of particles is accounted for the different positions of the beads in the corresponding phase contrast and fluorescent pictures The arrows in the phase contrast pictures point at vacuoles where the FITC-beads are arrested in *sodC*$^-$ cells.

Figure 4

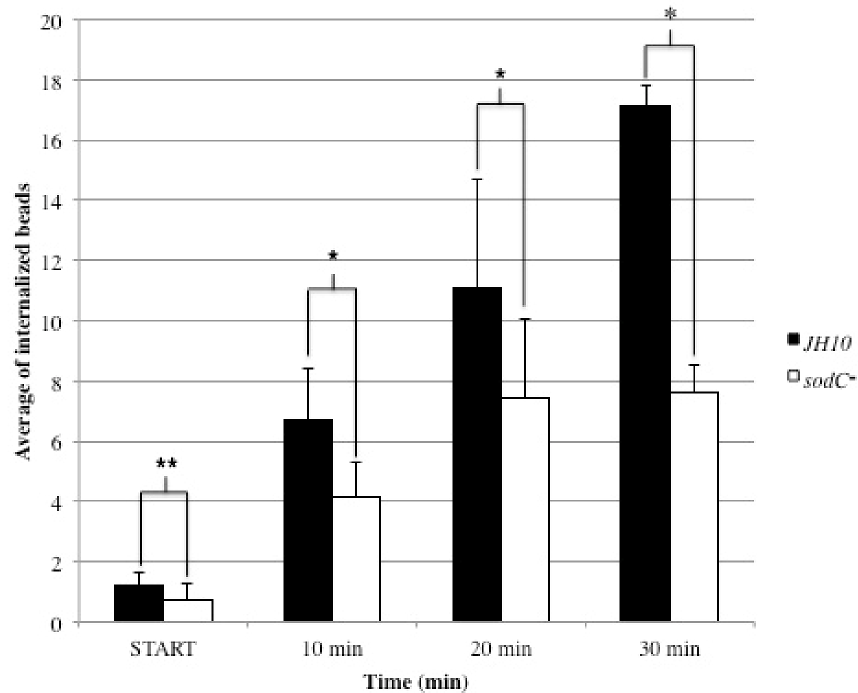

Figure 4: Particle uptake by wild type and *sodC⁻* *Dictyostelium* cells

The average particle uptake of three sets in both wild type and *sodC*⁻ *Dictyostelium* cells can be seen though different feeding times. The start period represents 0-2 minutes of contact between FITC- latex beads and wild type and *sodC*⁻ cells before spinning (see Methodology). The difference in particle uptake between wild type and *sodC*⁻ cells shows a P value <0.05 at the initial START point and a P value<0.01 at all other time points. Particle uptake is significantly less in *sodC*⁻ *Dictyostelium* cells. * = P value<0.01, ** = P value < 0.05.

Figure 5

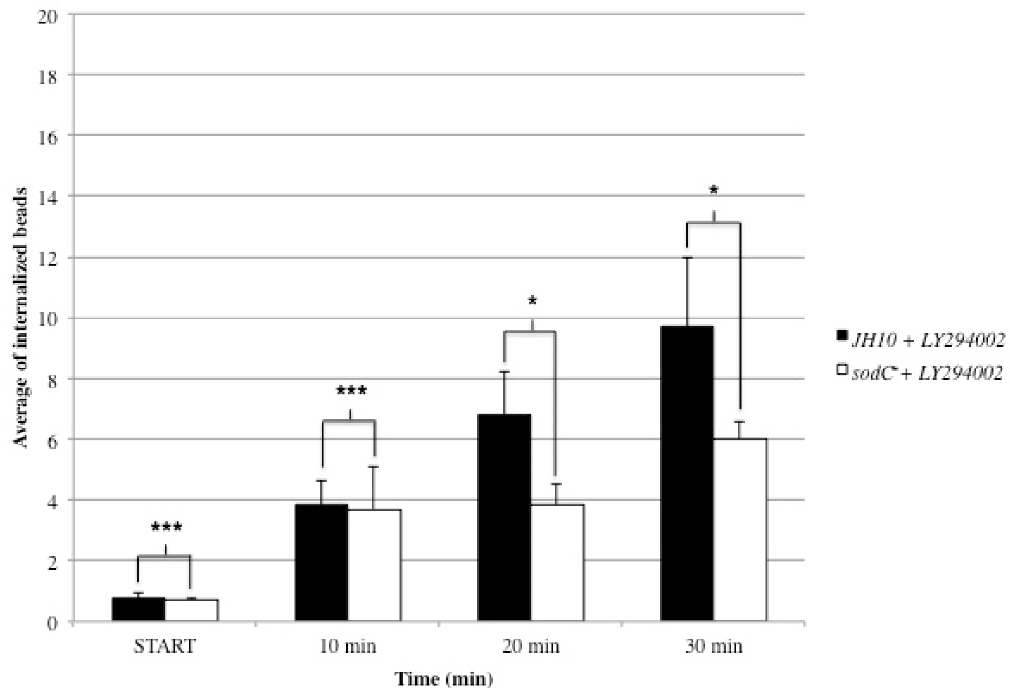

Figure 5: Particle uptake by wild type and *sodC⁻ Dictyostelium* cells after addition of the PI3K inhibitor LY294002

The average particle uptake of three sets in both wild type and *sodC⁻ Dictyostelium* cells after the addition the PI 3-kinase inhibitor LY294002 can be seen at different feeding times. The start period represents 0-2 minutes of contact between FITC- latex beads and wild type and *sodC⁻* cells, respectively, before spinning (see Methodology). Wild type cells treated with LY294002 show significantly decreased (~ 40%) particle uptake over the time course while *sodC⁻* cells generally exhibit similar level particle uptake over the assay period. * = P value<0.01, *** = P value >0.05.

Figure 6

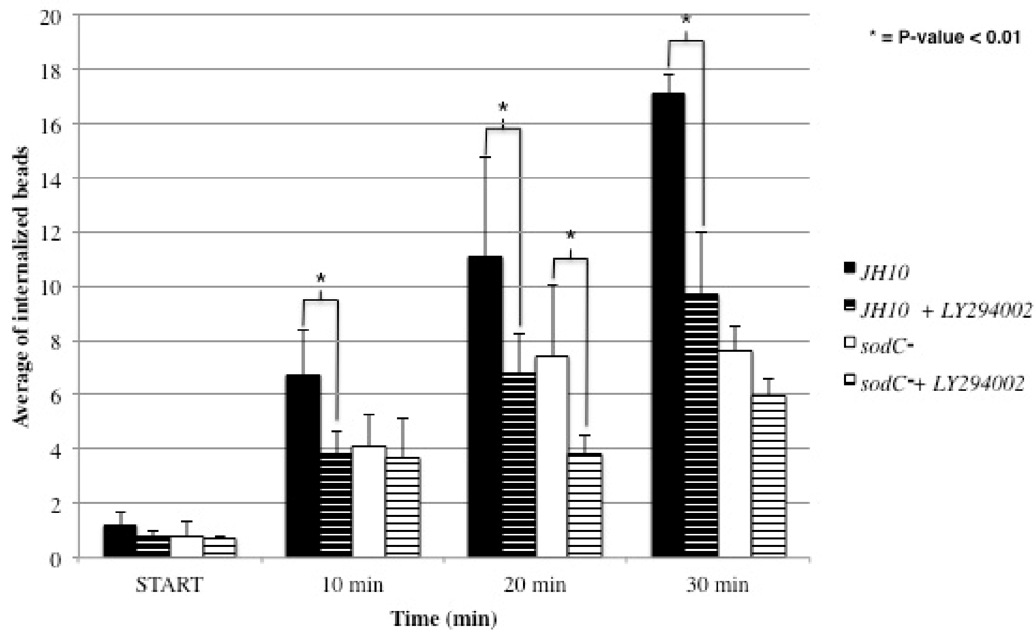

Figure 6: Comparison of particle uptake in wild type and *sodC⁻ Dictyostelium* cells before and after the addition of the PI3K inhibitor LY294002

The average particle uptake of three sets in both wild type and *sodC⁻ Dictyostelium* cells before and after the addition the PI 3-kinase inhibitor LY294002 can be seen at different feeding times. The start period represents 0-2 minutes of contact between FITC- latex beads and wild type and *sodC⁻* cells, respectively, before spinning (see Methodology). Wild type cells treated with LY show significantly decreased (~ 30%) particle uptake over the time course when compared to wild type cells not treated with LY. *sodC⁻* cells treated with LY generally exhibit similar level particle uptake over the assay period when compared to *sodC⁻* cells not treated with LY. * = P value<0.01.

Figure 7

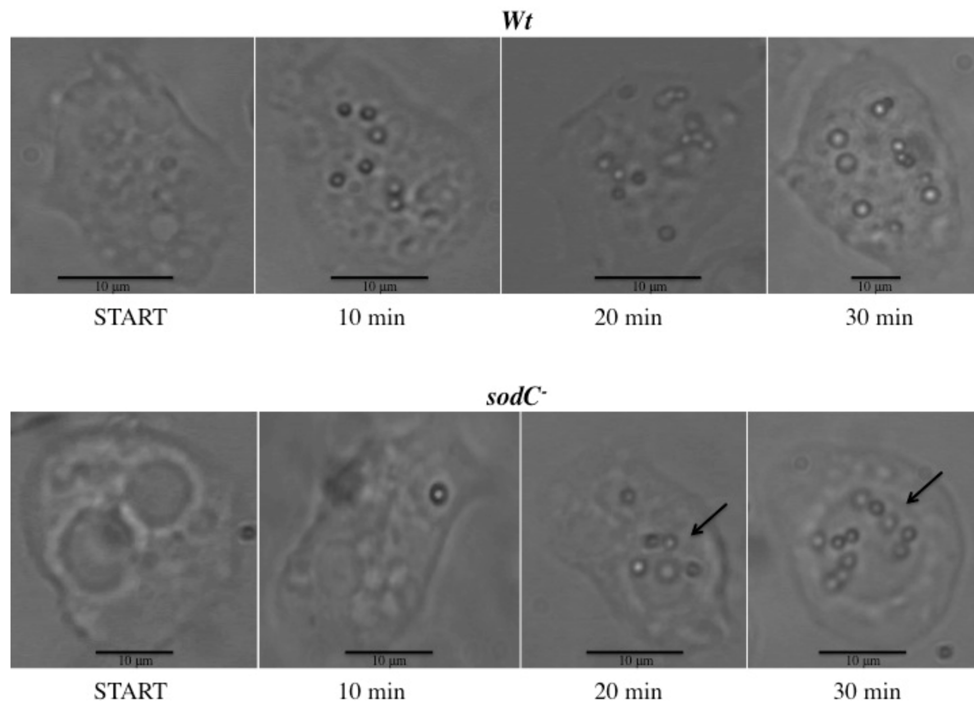

Figure 7: Particle uptake in wild type and *sodC- Dictyostelium* cells after addition of the PI3K inhibitor LY294002

This figure shows a decrease in particle in both wild type and *sodC*$^{-}$ cells after treatment with LY. The top pictures are representative phase contrast pictures of wild type cells chose during the different time points in the particle uptake assay. The bottom pictures are representative phase contrast pictures of *sodC*$^{-}$ cells. The start period represents 0-2 minutes of contact between FITC- latex beads and wild type and *sodC*$^{-}$ cells before spinning (see Methodology). The arrows in the 20min and 30min phase contrast pictures of *sodC*$^{-}$ cells point at vacuoles where the FITC-beads are arrested in *sodC*$^{-}$ cells.

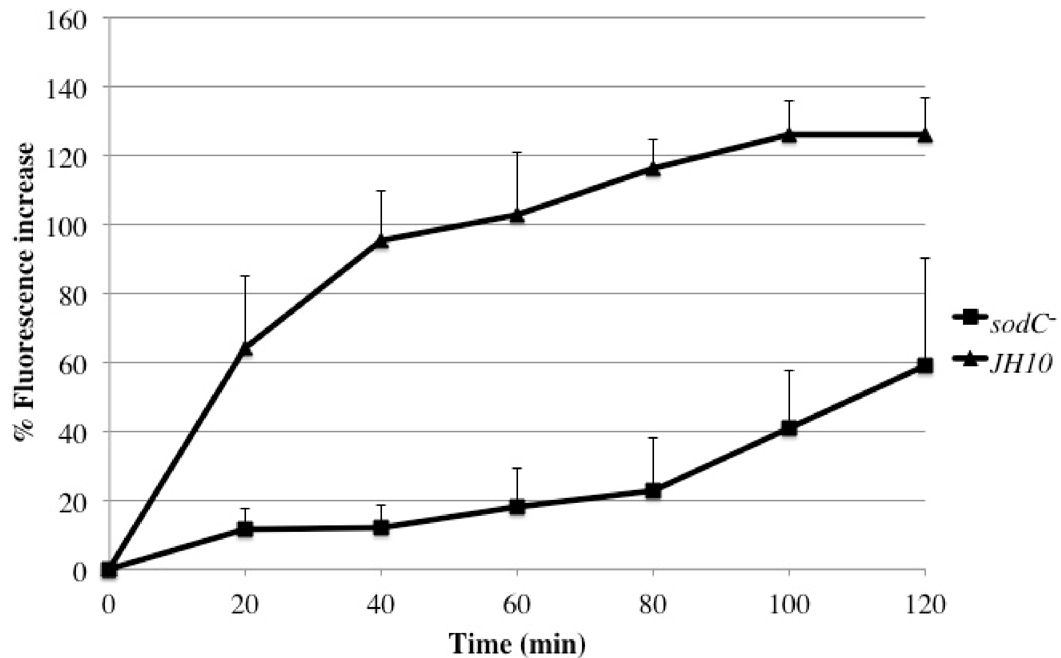

Figure 8: Exocytosis of FITC-dextran in wild type and *sodC*⁻ *Dictyostelium* cells

Differences in fluid exocytosis can be observed between wild type and *sodC*⁻ cells. Cells were incubated with fluorescent (FITC)-dextran (Mr) 70,000 up to 3 hours (see Methodology) and fluid exocytosis was subsequently measured every twenty minutes. Time zero represents the fluorescence of the extracellular medium after the initial three-hour incubation. At time 0min, fluorescence was designated as 0% and all subsequent measurements were converted as a percent increase compared to the value at time 0min. Over the time course of the assay, wild type cells displayed a steady increase in fluid exocytosis that seemed to reach a plateau after 100 minutes of exocytosis. Conversely, *sodC*⁻ cells started exocytizing significantly ~25% faster after 80 minutes of exocytosis.

Chapter 5: Discussion

Superoxide in both phagocytic and nonphagocytic cells plays a number of critical functions beyond the classical bacteriocidal function in the phagosome. NADPH oxidases (Nox), is responsible for the significant portion of cellular superoxide production, localize not only in the plasma membrane and phagosomes but also in intracellular endosomes. Mammalian Nox2, for example, exhibit plasma membrane and phagosomal localizations, upon which it mediates extracellular superoxide production. Nox1, in contrast, exhibits intracellular endosomal localization. Superoxide generation in vascular cells occurs in the early endosome and requires the expression of chloride channel 3 (CLC3), which is a broad specificity anion channel that could support superoxide transport from endosome to cytoplasm, it was suggested that Nox2-generated endosomal superoxide radicals transport to the cytoplasm through CLC3 (Miller *et al.*, 2007; Lassegue, 2007; Brown and Griendling, 2009).

Given that superoxide radicals can be detrimental to the host, it is highly likely that cells not only tightly control the production of the superoxide, but also regulate the endosomal levels of the radical. Among the three types of Superoxide Dismutases, the extracellular superoxide dismutase, EC-SOD, is likely to play a host protective role in the endosomes. Other SOD proteins are either mitochondrial or cytoplasmic. A recent study demonstrated that EC-SOD does indeed localize in the endosomes, while a naturally occurring genetic variant, EC-SODR213G, fails to do so (Chu *et al.*, 2006). The goal of the study was to determine if EC-SOD localize to the nucleus, but the endosomal EC-SOD proteins did not translocate to the nucleus and so the function of endosomal EC-

SOD is not known. *Dictyostelium* cells also contain multiple types of SOD protein including the extracellular one, SodC (Veeranki, *et al.,* 2008). *Dictyostelium* SodC localizes on the plasma membrane (Veeranki *et al*., 2008), but the follow up study showed that cells lacking SodC (*sodC*$^{-}$ cells) display intracellular vesicular accumulation of superoxide radicals and a version of GFP fused SodC localized intracellular vesicular compartment (unpublished data, Kim Lab).

Earlier studies on *sodC*$^{-}$ cells disclosed its role in directional cell migration through regulating redox sensitive small GTPase RasG (Veeranki *et al.,* 2008). Furthermore, a study showed that cells expressing constitutively activated mutant RasG(G12T) increased Cell-Substratum Adhesion, but decreased filopodia formation and phagocytosis (Chen and Katz, 2000). Consistent with the previous RasG (G12T) overexpression study, *sodC*$^{-}$ cells, which display high basal RasG activity, were significantly impaired in particle uptake. The current study, however, uncovered that not only the efficiency of particle uptake is decreased in *sodC*$^{-}$ cells, but also the fate of the internalized particles was completely derailed. Instead of joining lysosomal pathway, particles in *sodC*$^{-}$ cells efficiently recruited into aberrant vacuoles. A study in our laboratory recently determined that these vacuoles lack lysosomal markers (unpublished data, Kim lab). Apparently, particles in these non-lysosomal vacuoles have escaped from the destruction fate and would have more options of establishing their relationship with *Dictyostelium* cells.

To further dissect the effect of superoxide on particle uptake, wild type and *sodC*$^{-}$ cells were treated with the PI3K inhibitor LY294002 that inhibits macropinocytosis but

not phagocytosis. As described earlier, wild type cells exhibited dramatic decrease in the particle uptake upon LY294002 treatment but no such effect was observed from *sodC*$^-$ cells treated identically, indicating that SodC is critical for proper macropinocytosis rather than phagocytosis. Considering that SodC is regulating RasG, which in turn controls PI3K activation and thus macropinocytosis, the aberrant hyperactivation of RasG in *sodC*$^-$ cells is likely the cause for the decreased particle uptake. It is, however, puzzling that PI3K inhibition did not improve particle uptake of *sodC*$^-$ cells, considering that LY294002 treated *sodC*$^-$ cells displayed excellent chemotaxis (Veeranki *et al*., 2008). It is thus likely that there exist additional defects that hamper particle uptake behavior of *sodC*$^-$ cells.

Lastly, *sodC*$^-$ cells also displayed significantly impaired exocytosis. *sodC*$^-$ cells not only showed lower level of fluid phase discharge, but also severely delayed discharge. Considering that cells were saturated with 3 hour FITC-dextran feeding, a rapid initial exocytosis is expected with delayed saturating phase as cellular interior is chased with non-fluorescent media. Wild type cells exactly displayed this pattern, but *sodC*$^-$ cells were resistant for exocytosis for an hour and then showed delayed yet lower level of discharge. This is likely that the convergence of not only the particles, but also the fluid uptake into large vacuoles in *sodC*$^-$ cells. With this saturated vacuoles, *sodC*$^-$ cells would experience much limited room for trafficking both particles and fluids.

This study, for the first time, demonstrated that proper metabolism of reactive oxygen species Superoxide is critical for efficient particle uptake, proper trafficking of the internalized particles, and exocytosis in *Dictyostelium* cells. Given that all the

components discussed here are conserved in higher eukaryotes including human, the current study will be highly informative for other disciplines of science such as pathogen-host interaction and symbiosis.

5.1.1 Relevance to mammalian cells

The process of macropinocytosis has recently emerged as a major endocytic mechanism in the cell entry of animal viruses (Mercer and Helenius, 2012) and it is known that pathogens such as *Legionella pneumophila* increase superoxide dismutase SodA (homologous to SodC) in early endosomes and that it is able to replicate in hosts that lack coronin due to improper superoxide generation at the phagosome (Shevchuck *et al*., 2009). Hence this study will be highly informative for pathogen-host interaction and symbiosis.

In addition to the importance of better understanding the role of superoxide dismutase in pathogen-host interaction during the early and later steps of endocytic processes, the exocytosis of eukaryotic vesicles has recently become of upmost importance in understanding drug detoxification and other cellular responses (Tatischeff, 2013). Exosomes were originally believed to be a waste reservoir, but through different studies, the roles of exosomes have been found to include transferring immunity in blood cells, behaving as pathogenic biomarkers, and mediating intracellular communication. The study of the regulation of exosomes during exocytosis has proven to be a difficult task in human cells due to the complexity of the different cell types; hence by studying a simple model system such as *Dictyostelium discoideum*, whose homology to cells of the

innate immune system makes them excellent systems to study biochemical pathways in neutrophils and macrophages, has become an ideal.

5.2 Future perspective

The misregulation of the macropinocytic pathway in *sodC*$^-$ cells is evident as the destination of the particles internalized by *sodC*$^-$ cells is different than in wild type *D. discoideum* cells. Instead of the particles progressing towards the early and late endosomes, latex beads in *sodC*$^-$ cells end up in vacuoles (Fig. 3 and Fig.7). Because the identity of this vacuoles is not well studied, by tracking some of the regulatory proteins known to be involved in macropinocytosis we could be able to understand better this aberrant pathway in *sodC*$^-$ cells.

Some of the GFP tagged regulatory proteins that could be utilized are: coronin (coronin-GFP), a construct with two tandem FYVE domains (2FYVE-GFP) from an early endosome that binds PI(3)P, and active Ras protein marker (GFP-RBD). Studies have shown that coronin is enriched in the phagocytic cups that form in response to particle attachment, that it accumulates in the phagocytic cups in less than one minute after particle attachment and that it is gradually released from the cups one minute after particle engulfment (Maniak *et al.,* 1995). Active Ras is an upstream regulator of PI3K (Sasaki *et al.,* 2004; Cox and Der, 2003; Heo and Campbell, 2005; Veeranki *et al*., 2008). One type of PI3K, Vps34, which is not activated by Ras, is responsible for most PI(3)P generated on phagosomes (Bohdanowicz and Grinstein, 2013). In addition to the role of PI(3)P in phagosome maturation, PI(3)P is essential for the generation of ROS within maturing phagosomes because it recruits subunits of the NADPH oxidase complex that is

required for phagosomes to generate superoxide (Bohdanowicz and Grinstein, 2013). By colocalization studies on coronin, the construct with the two FYVE domains, and active Ras we could be able to track the phagosomes from particle uptake to degradation or exocytosis in *Dictyostelium discoideum* cells lacking superoxide dismutase C and in wild type.

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