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Characterization of phosphatidylinositol (3,4,5)-trisphosphate subcellular localization during endocytosis in *Entamoeba histolytica*

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CHARACTERIZATION OF PHOSPHATIDYLINOSITOL (3, 4, 5)-TRISPHOSPHATE SUBCELLULAR LOCALIZATION DURING ENDOCYTOSIS IN ENTAMOEBA HISTOLYTICA

A Thesis
Presented to
the Graduate School of
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In Partial Fulfillment
of the Requirements for the Degree
Master of Science
Biological Sciences

by
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Accepted by:
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Dr. Kimberly S. Paul
Dr. Bradley M. Hersh
ABSTRACT

*Entamoeba histolytica* is a protozoan parasite that causes amoebic dysentery and liver abscess. Currently, the World Health Organization estimates that amoebic dysentery results in 40,000-100,000 deaths annually worldwide, which places amoebiasis second only to malaria as a leading cause of death due to parasitic infection. The pathogenesis of invasive amoebiasis depends on the parasite’s ability to carry out endocytosis in the host’s bowel lumen enabling it to colonize the digestive tract. The pathogen is contracted by the ingestion of its multinucleate cysts. Upon excystation in the small intestine, motile trophozoites are released into the bowel lumen, where bacteria, erythrocytes, and cellular debris serve as their food source. Phagocytosis in *E. histolytica* has been directly linked to its ability to destroy host tissues. Thus, understanding the molecular mechanisms of cell movement and nutrient uptake will provide insight into factors that contribute to the pathogen’s virulence and reveal potential molecular targets for interventions.

The goal of this Master’s thesis research project is to examine the role of a second messenger lipid, phosphatidylinositol (3,4,5)-trisphosphate (PIP₃), during endocytosis in *E. histolytica* trophozoites. Phosphoinositides (PIs) represent a dynamic pool of membrane-associated lipids, whose composition is regulated by several isoforms of phosphatidylinositol 3-kinase (PI 3-kinase). A number of recent studies have implicated PIs in regulating virulence-associated cellular
functions in *E. histolytica*. For instance, a putative PI3-kinase was identified among the signaling molecules that were physically associated with *E. histolytica* phagosomes, and pharmacological inhibition of PI3-kinase blocked the uptake of erythrocytes by this amoeba. Moreover, a singly phosphorylated product of PI3-kinase activity, PI3P, was shown to accumulate in the erythrophagosomal cups in *E. histolytica*. These observations provided the incentive to investigate the distribution of PIP$_3$ during endocytosis in *E. histolytica*. We implemented two approaches utilizing GST- and GFP-tagged biosensors to examine the spatial and temporal aspects of PIP$_3$ distribution during endocytosis in *E. histolytica*.

We have shown that PIP$_3$ accumulated in pseudopodia and erythrophagosomal cups but not in fluid phase pinosomes in *E. histolytica* trophozoites. We also observed high steady-state levels of PIP$_3$ in the plasma membrane of *E. histolytica* trophozoites, which were not abolished by serum withdrawal. To our knowledge, this is the first report describing PIP$_3$ distribution during phagocytosis in *E. histolytica* that also elucidates a unique aspect of biology of this parasite, namely the stability of PIP$_3$ lipid in plasma membrane. This distinguishes the physiology of this parasite from that of mammalian host cells. This study provides insight into the molecular mechanisms of *E. histolytica* pathogenicity, since nutrient uptake represents an important virulence function in this parasite.
DEDICATION

This work is dedicated to my parents, Alexey and Svetlana, whose unwavering faith in me and relentless encouragements have helped me to pursue my dreams. To my little brother, Constantine, who taught me to be patient and to whom I owe my enduring interest in medicine and science.
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It is a pleasure for me to acknowledge the contribution of my teachers and committee members, Kimberly S. Paul and Bradley M. Hersh, to my graduate experience. I am grateful to Dr. Paul for her fascinating lectures and discussions about infectious diseases. But I am especially thankful for her insight into science and academic life in general and for long and pleasant conversations with me on topics ranging from career choices to medical ethics. I want to thank Dr. Hersh for not only teaching me the principles and subtleties of developmental and evolutionary biology, but also for helping me to assemble my previously fragmented knowledge of biology into a wholesome understanding of natural processes. I am also grateful to him for challenging my scientific writing skills and helping me improve my analytic and synthetic abilities thus bolstering my
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CHAPTER 1
LITERATURE REVIEW

*Entamoeba histolytica*

**Background and Epidemiological Significance**

*Entamoeba histolytica* is an intestinal protozoan parasite that causes amoebic dysentery (intestinal disease) and liver abscess. Currently, the World Health Organization estimates that amoebic dysentery results in 40,000-100,000 deaths annually worldwide, which places amoebiasis second only to malaria as a leading cause of death due to protozoan parasitic infection (106). Although *E. histolytica* infection is one of the major contributors to morbidity and mortality in developing countries, accurate global prevalence and incidence data on amoebiasis is currently unavailable. This is largely attributed to the inability of earlier diagnostic methods to distinguish between *E. histolytica* and a closely related non-pathogenic amoeba, *E. dispar* (46). The development of novel molecular diagnostic approaches created opportunities for the reassessment of old epidemiological data on invasive intestinal amoebiasis worldwide. For instance, in Bangladesh, where 1 in 30 children dies from diarrheal diseases, anti-*E. histolytica* antibodies were detected in almost 50% of children younger than six years of age (38). Unexpectedly, a high burden of *E. histolytica* infection was found in Egypt, where 38% of people with acute diarrhea presented with amoebic dysentery (1). In Mexico, the National Serosurvey estimated that
infection with *E. histolytica* resulted in 1 million cases of invasive amoebiasis and 1,000 deaths per annum (14). Therefore, *Entamoeba* infection is a serious public health problem in developing countries with low basic hygiene standards and the lack of water sanitation. Since *E. histolytica* is mainly a human parasite, improvements in sanitation can help prevent the fecal-oral spread of this pathogen and potentially eradicate the disease. However, overpopulation, tropical climate, and scarce water availability in developing countries impede progress in this area and make eradication of amoebiases unlikely in the foreseeable future (13; 81).

Although *E. histolytica* infection is most prevalent in developing countries, amoebiasis presents a growing concern in certain high-risk populations in industrialized countries. Recent studies found that international travelers to tropical and subtropical countries, adopted children and immigrants from endemic areas, patients suffering from immunodeficiency due to HIV infection, men who engage in homosexual behavior, and institutionalized individuals are at an elevated risk of contracting and transmitting *E. histolytica* infection (31; 44; 73). Moreover, since *E. histolytica* is a food- and waterborne pathogen with a high potential for mass dissemination, the National Institute of Health (NIH) has classified it as a Category B priority bioterrorism agent. Therefore, currently there is an elevated need for the development of improved prevention, diagnostic, and treatment methods for amoebic dysentery caused by *E. histolytica*, which
demands an in-depth understanding of basic biology and pathogenicity of this parasite.

**Transmission and Pathogenesis**

*E. histolytica* is commonly contracted by the ingestion of fecally contaminated food and water that contain the parasite’s multinucleate infective cysts (89). The majority of infected individuals, however, do not develop an invasive disease and become asymptomatic cyst passers, thus contributing to the perpetual propagation of this parasite in the human population. Only 1 in 10 individuals infected with *E. histolytica* develops invasive amoebiasis presenting with abdominal pain, bloody diarrhea, and dehydration all of which gradually develop over the course of several weeks (73). The colonization of the human intestine with *E. histolytica* begins with excystation in the small intestine and the release of motile trophozoites into the bowel lumen, where bacteria and cellular debris initially serve as their food source. Once excystation is complete, the trophozoites recognize N-acetyl-D-galactosamine (GalNAc) on the surface of host endothelial cells and adhere to the intestinal wall via the surface protein, Gal/GalNAc-inhibitable lectin. Subsequently, trophozoites initiate the destruction of colonic epithelial cells in a contact-dependent fashion by inducing apoptosis in the host cells and then phagocytosing them. Moreover, *E. histolytica* releases lytic molecules, which degrade the extracellular matrix and facilitate the invasion of host tissues by the parasite. The destruction and invasion of the intestinal epithelium and underlying layers by *E. histolytica* results in the formation of
characteristic “flask-shaped” ulcers, with trophozoites populating the base of the ulceration (84). Occasionally, \textit{E. histolytica} disseminates via the hematogenous route to extraintestinal sites, with the liver being the most common site of colonization and amoebic abscess formation. Among other life-threatening complications of intestinal amoebiasis are toxic megacolon (acute dilation of the colon), ulcer perforation and peritonitis, amoebomas (the expansion of granular tissue into intestinal lumen resembling a tumor), and fistula formation (46).

\textbf{Diagnosis and Treatment}

Historically, \textit{E. histolytica} is diagnosed by the light microscopic examination of stool samples and identification of motile trophozoites or multinucleate cysts. This method, however, fails to differentiate between two closely related and morphologically indistinguishable \textit{Entamoeba} species, \textit{E. histolytica} and the non-pathogenic \textit{E. dispar}. Since non-pathogenic amoebae are not capable of erythrophagocytosis and invasion of host tissues, the presence of ingested red blood cells in the microscopic stool preparations is usually indicative of \textit{E. histolytica} infection. The use of erythrophagocytosis as a main diagnostic feature of invasive amoebiasis is hindered, however, by the low sensitivity of this technique (46). A diagnostic method that is both highly specific and sensitive to \textit{E. histolytica} involves the isolation and culture of trophozoites with subsequent zymodeme analysis (46). Although this technique is considered the ‘gold standard’ for the diagnosis of invasive amoebiasis, it is both time-consuming and labor-intensive, undermining its utility as an effective diagnostic tool in the field.
Recent advances in molecular biology allowed the development of two novel diagnostic methods that accurately and rapidly detect *E. histolytica* with high sensitivity. The detection of *E. histolytica*–specific antigens and small ribosomal RNA genes (rRNA) in blood and stool samples using commercially available ELISA tests and PCR are currently widely used to diagnose *E. histolytica* infection (46; 89).

In 1997 the World Health Organization, Pan American Health Organization, and United Nations Educational, Scientific and Cultural Organization (UNESCO) issued a joint report containing recommendations on treatment strategies of amoebiasis (106). According to this report, the asymptomatic carriage of *Entamoeba* spp. should be treated only in cases of confirmed *E. histolytica* infection. Invasive amoebiasis is treated with tissue amoebicides such as metronidazole or tinidazole, followed by luminal amoebicide, paromomycin, to ensure the clearance of intraluminal parasites. Asymptomatic amoebic infections are treated exclusively with luminal amoebicides. Although antibiotic therapies proved fairly successful in the past, some studies report incomplete resolution of *E. histolytica* infection and frequent re-infection (53). In addition, the emergence of drug-resistance has been shown in *E. histolytica* grown *in vitro* (65), which further demonstrates the need for the development of new preventative strategies against *E. histolytica*.

Recent serological studies in Bangladeshi children revealed a correlation between the presence of mucosal IgA anti-lectin antibodies and immune
protection against *E. histolytica* infection (39). This finding, together with the observation that after a diarrheal episode associated with *E. histolytica* children were almost 50% less likely to develop amoebiasis (40), provide evidence for the existence of naturally acquired immunity to *E. histolytica*, as well as hope for the development of anti-amoebic vaccine. Currently, several vaccine trials are successfully exploring the potential of Gal/GalNAc-inhibitable lectin to serve as a vaccine target due to its highly conserved structure, antigenic properties, and cell-surface location (42). The search for other *E. histolytica* immunogens is currently underway. However, gaps in our understanding of the basic biology of this parasite are the main barriers to vaccine and new drug treatment developments. Therefore, research into the molecular mechanisms of such important *E. histolytica* virulence functions as adherence, secretion, host killing, phagocytosis, and critical signaling pathways may reveal potential molecular targets for effective interventions.

**Endocytosis as Important Virulence Function**

*Pinocytosis*

A notable morphological feature of *E. histolytica* that is apparent upon the examination of trophozoites with light or electron microscopy is its unusually high degree of cytoplasmic vacuolization (Fig. 1.1 A). This extensive network of vesicular intracellular compartments provided the first evidence that *E. histolytica* possesses a robust capacity for endocytosis, a plasma membrane-associated process in which a eukaryotic cell engulfs extracellular fluids or particles (87). *E.*
*E. histolytica* is known to take up the amount of fluid corresponding to ~15% of the total cell volume at equilibrium (3). The process by which the uptake of exogenous fluids and solutes occurs is commonly referred to as pinocytosis. *E. histolytica* grown in axenic culture utilizes pinocytosis as a primary mode of nutrient acquisition (59). The pinocytic pathway was delineated in *E. histolytica* using fluorescent fluid-phase markers such as FITC-dextran, which are readily taken up by the trophozoites. Pinocytosis in *E. histolytica* does not exhibit any spatial restriction and can take place virtually anywhere on the cell surface. It occurs in a constitutive fashion ensuring a continuous sampling of the environment by the protozoan. The uptake of fluid cargo begins with the formation of pinosomal invaginations in the plasma membrane (Fig. 1B and C) or extension of small actin-driven pseudopodia around larger fluid aliquots (59). Subsequent closure of pinocytic vesicles leads to the formation of early endosomes of assorted sizes, which are rapidly acidified, probably by the incorporation of vacuolar H⁺-ATPases (59). That endocytic compartments merge with secretory vesicles is evidenced by the enrichment of acid phosphatases and several cysteine proteases in both early and late endosomal vesicles (94). Progressive maturation of endocytic compartments is accompanied by the pH neutralization of their contents. The subsequent fate of endosomal vesicles varies, with some endosomes being recycled or fused with other endosomal compartments, while others becoming candidates for exocytosis. On average,
the transit of fluid-filled vesicles in *E. histolytica* requires approximately two hours (59).

**Figure 1.1.** Electron micrographs of *E. histolytica* grown in axenic cultures. (A) Transmission electron micrograph of *E. histolytica* trophozoite depicting a highly vacuolated cytoplasm. Intracellular vesicles of assorted sizes represent micro- and macropinosomes that form as a result of fluid phase uptake by this parasite. (B) Scanning electron micrograph of *E. histolytica* cell surface. Numerous pore-like openings on the cell surface correspond to pinosomal invaginations. (C) A close-up of two pinosomal pits on the surface of *E. histolytica* trophozoite.

While pinocytosis plays an important physiological role in nutrient uptake, environmental sensing, and plasma membrane turnover in axenically cultured *E. histolytica*, it is also a critical virulence function in this parasite. Among the first observations that highlighted the importance of pinocytosis in the pathogenicity of this amoeba are marked differences in the axenic growth potential between *E.*
E. histolytica and its non-pathogenic relative, E. dispar. While E. histolytica is successfully grown in axenic medium (in the absence of other microorganisms as a food source), the efforts to cultivate E. dispar under the same conditions have proven unproductive (72). It is suspected that E. dispar capacity for pinocytic uptake is inferior to that of E. histolytica. These observations are further corroborated by scanning and transmission electron microscopy of E. dispar, which revealed a smooth cell surface practically devoid of pinocytic pits and many fewer small intracellular vesicles (26).

Bacterial uptake and virulence

Another endocytic process, which has been directly linked to virulence in E. histolytica, is phagocytosis. When in the lumen of a large intestine, E. histolytica relies on bacteria, erythrocytes and cellular debris as their main food source. Numerous studies have demonstrated a direct correlation between E. histolytica phagocytic ability and its virulence. Pioneering studies on E. histolytica virulence factors found that ingestion of intact bacteria can markedly augment trophozoite virulence as indicated by the increased rate of target cell monolayer destruction (12). These early studies suggested that bacteria might transfer some proteinous factors to E. histolytica that alter its virulence responses (12; 105). Recent transcriptional profiling of E. histolytica exposed to Escherichia coli showed that bacteria induced a significant upregulation of protein kinase, ABC transporter, Rho family GTPase, and hsp90 gene expression (22), which may collectively contribute to the augmentation of virulence in this parasite.
Apoptotic host cell killing and phagocytosis

The name of this enteric protozoan, ‘*histolytica*’, accurately reflects one of its most prominent pathognomic attributes, its “tissue destroying ability” (53). *E. histolytica* is one of the few organisms that possesses a large arsenal of cytotoxic and lytic molecules that facilitates tissue destruction and invasion by this parasite. For example, *E. histolytica* secretes a unique pore-forming molecule, amoebapore, which is capable of disrupting bacterial and host cell membranes (53). Moreover, this parasite is equipped with potent degradative enzymes such as acid phosphatases and cysteine proteases that disrupt intercellular adhesion complexes and degrade host extracellular matrix resulting in the destruction of colonic epithelium and its underlying tissues.

Recent studies have demonstrated that *E. histolytica*-induced cytotoxicity is intimately linked with phagocytosis. It is known that *E. histolytica* triggers cytopathic effects in host cells in a contact-dependent fashion involving the Gal/GalNAc-inhibitable lectin (24). Huston *et al.* demonstrated that contact-dependent cell killing is initiated via the activation of host cell caspase-3 followed by apoptotic death of target cells (47). Subsequently, this group has shown that apoptotic host cell killing preceded and facilitated amebic phagocytosis (45). Furthermore, Bailey *et al.* directly demonstrated that exogenous liposomes containing phosphatidylserine, a molecule commonly exposed on the surface of apoptotic cells, induce the reorganization of actin cytoskeleton and phagocytosis in *E. histolytica* (7). Based on these observations, it has been proposed that
phagocytosis of apoptotic target cells by *E. histolytica* serves as a mechanism of evasion of the host’s immune system, since engulfment of cellular debris limits the spillage of toxic intracellular components and restricts host inflammatory response (45). Therefore, contact-dependent killing of host target cells, followed by their phagocytosis, is a prerequisite for tissue invasion and establishment of chronic infection by this parasite.

*Erythrophagocytosis*

Traditionally, the uptake of human red blood cells (hRBCs) by *E. histolytica*, also known as erythrophagocytosis, has been an important diagnostic criterion to distinguish invasive amoebiasis from the intestinal carriage of non-pathogenic commensals (106). Indeed, non-invasive enteric amoebae, *E. dispar* and *E. moshkovskii*, isolated from the dysenteric stool samples, rarely showed indication of erythrophagocytosis (34; 95). The direct relationship between *E. histolytica* ability to ingest erythrocytes and its virulence has been demonstrated in a number of studies both *in vitro* and *in vivo*. Orozco *et al.* were among the first to show that phagocytosis-deficient clones of *E. histolytica* (L-6) exhibited reduced virulence as evidenced by the inability of these trophozoites to produce abscesses in hamster livers (64). Moreover, the recovery of pathogenicity in *E. histolytica* L-6 trophozoites was associated with the recuperation of erythrophagocytosis. The characterization of erythrophagocytosis-deficient *E. histolytica* mutants obtained through chemical mutagenesis revealed similar reduction in *in vivo* virulence (75). Taken together, these observations
demonstrate that erythrophagocytosis plays a critical role in the pathogenicity of *E. histolytica* and progression of the invasive disease.

Mammalian erythrocytes are routinely used in the laboratories to study the physiological and molecular mechanisms of phagocytosis in *E. histolytica*. Erythrophagocytosis begins with the adherence of *E. histolytica* to the erythrocytes, which has been shown to be dependent on the coordinated function of the Gal/GalNAc-inhibitable lectin and an unidentified phosphotidylserine coreceptor (10). Upon contact with hRBCs, *E. histolytica* induces morphological deformations of the erythrocyte cell surface. The blood cells take on a crenulated appearance, characterized by membrane blebbing, similar to the changes triggered by the exposure to calcium ions. This cell surface distortion has been attributed to the exposure of phosphotidylserines on the outer leaflet of erythrocyte membrane (10). Moreover, this study found that phosphotidylserine exposure and physical deformation of the erythrocyte surface facilitated their uptake by the amoebae. Modified erythrocytes are rapidly engulfed by *E. histolytica* as intact particles. Two studies of erythrophagocytosis in *E. histolytica* consistently reported that, on average, each trophozoite is capable of engulfing eight erythrocytes after a 5-min exposure (60; 66). It is not uncommon to observe several erythrophagocytic events occurring simultaneously on a single trophozoite. Phagocytosis of hRBCs may involve the extension of pseudopodia around the adhered particle followed by the closure of the phagocytic membranes and internalization of the nascent phagosome. Alternatively,
erythrocytes appear to be “sucked in” by the trophozoites through narrow cytoplasmic channels, which does not involve the extension of pseudopodia (102). The mechanism and kinetics of intracellular degradation of hRBCs by *E. histolytica* was investigated by Mora-Galindo *et al.* using electron microscopy, electrophoresis, and X-ray spectroscopy (60). They proposed a three-stage model to describe the sequence of events during erythrocyte digestion by the parasite. According to this model, stage 1 corresponds to the intact erythrocyte trapped inside an early phagosome. During stage 2, the degradation of the erythrocyte cytoplasm is initiated with its plasma membrane remaining intact. Finally, stage 3 corresponds to numerous small digestive vacuoles filled with either membranes and/or amorphous electrondense particles. The completion of hemoglobin digestion required approximately 3 hours, while digestion of the entire erythrocyte occurred over 9-12 hours after the challenge. This study also demonstrated that amebic cysteine proteases are required for the intracellular erythrocyte degradation in *E. histolytica*.

While physiological mechanisms of erythrophagocytosis are well understood, the molecular basis of this important virulence function in *E. histolytica* is currently under investigation. Recent completion of *E. histolytica* genome sequence and advances in large-scale proteomic analyses greatly expedited the discovery of important molecules involved in phagocytosis in this parasite. The next section of this chapter is dedicated to the review of molecules and signaling pathways involved in phagocytosis in *E. histolytica*. 
Molecular Mechanisms of Phagosome Biogenesis

The role of the Gal/GalNAc-inhibitable lectin in the initiation of phagocytosis of human enterocytes and red blood cells by *E. histolytica* has been known for some time and continues to be thoroughly investigated. Recent proteomic analysis of isolated *E. histolytica* phagosomes revealed an array of other amoebic surface molecules that may potentially mediate the early stages of phagocytosis (58; 63). For instance, two membrane proteins, *glycoprotein 1 and 6*, were isolated from *E. histolytica* early phagosomes by proteomic analysis and were shown to contain an ABC-type transporter domain (57). The significance of this finding becomes evident in the view of recently described ABC transporter function as a regulator of cell membrane lipid arrangement, which may promote cellular phagocytic activity (37). Another putative candidate that might be involved in the adhesion of *E. histolytica* to apoptotic host cells is a surface antigen *M17*, which was implicated in phosphotidylserine binding in other systems (57). A recent antibody screen for potential receptors involved in *E. histolytica* phagocytosis identified a novel serine-rich *E. histolytica* protein (SREHP), which can bind apoptotic corpses and initiate their phagocytosis (93).

Adhesion to bacteria, erythrocytes, and apoptotic host cells triggers a cascade of signal transduction events in *E. histolytica* that causes the reorganization of the actin cytoskeleton resulting in pseudopodia extension and subsequent phagocytosis. Two independent large-scale proteomic screens of isolated phagosomes in *E. histolytica* identified a multitude of molecular
components that might function as mediators of outside-in signaling leading to actin remodeling associated with phagosome biogenesis (58; 63). Among the best-characterized signaling proteins involved in phagocytosis in other systems is the superfamily of small GTP-binding proteins, which includes Rab, Rho/Rac, and Ras GTPases (77). Low molecular weight GTPases are known to be key regulators of actin remodeling and vesicle trafficking, which are central to the processes of endocytosis and cell movement (53). GTPases act as ‘molecular switches’ that regulate the docking and fusion of vesicles in the endocytic and secretory pathways by alternating between an active GTP-bound state and an inactive GDP-bound form. Due to their low intrinsic GTPase activity, Rab and other small GTPases rely on other regulatory elements such as GTPase activating proteins (GAPs), guanine nucleotide exchange factors (GEFs), and guanine nucleotide dissociation inhibitors (GDIs) for adequate GTP/GDP cycling (77). There are approximately 90 genes that encode putative EhRab GTPases as indicated by the analysis of recently completed E. histolytica genome (77). Two proteomic screens collectively identified 14 Rab-like GTPases that physically associated with E. histolytica phagosomes, including EhRabB, EhRab5, and EhRab7 (58; 63). Functional characterization of EhRabB revealed that it accumulated at the erythrophagocytic mouths and was mislocalized in the phagocytosis-deficient E. histolytica clone L-6 (35; 74). Not unexpectedly, isolation of bead-containing phagosomes in E. histolytica revealed the presence of EhRab5 and EhRab7. The role of EhRab5 in the uptake of gerbil RBCs was
previously demonstrated in *E. histolytica* mutants overexpressing HA-tagged Rab5 (79), and *EhRab7* was shown to be enriched in early and late endocytic compartments in *E. histolytica* (94; 101). More recent studies detected both *EhRab5* and *EhRab7* in the pre-phagosomal vacuoles, a unique endosomal organelle in *E. histolytica*, which is thought to deliver digestive enzymes to maturing phagosomes (78).

In addition to Rab GTPases, members of Rac- and Ras-family of small GTPases were detected in *E. histolytica* phagosomes including RacG and p21\textsuperscript{racA} (58). The mechanism of action of Rac GTPases is well studied in mammalian systems and involves activation of actin reorganization via interaction with the serine/threonine kinase, p65PAK, and indirect activation of SCAR/WAVE proteins. PIP\textsubscript{3} is an essential cofactor responsible for Rac GTPase cell membrane targeting (70). In *E. histolytica*, a pleckstrin homology (PH) domain-containing RacG was shown to be directly activated by RhoGEF1 (2) and was found enriched around erythrophagocytic vacuoles (58). Another important regulator of actin dynamics, a Ras-like GTPase p21\textsuperscript{racA}, was implicated in regulating the uptake of bacteria and hRBCs in *E. histolytica* since trophozoites overexpressing hyperactive RacA exhibited impaired phagocytosis (33). Interestingly, a putative downstream target of Rac GTPases, p21-activated kinase (EhPAK), which is known for its potent ability to promote actin polarization, was also identified in *E. histolytica* phagosomes (58). Functional studies involving overexpression of a constitutively active EhPAK showed a
marked increase in the rate of formation of nascent pseudopodia and hRBC phagocytosis (51). Taken together, these studies are beginning to decipher Rab- and Rho/Rac-dependent signal transduction pathways that lead to actin reorganization during phagocytosis in *E. histolytica*.

Currently, little is known about the upstream processes that regulate activation of RhoGEFs and small GTPases in *E. histolytica*. Recent studies in mammalian cells demonstrated the involvement of tyrosine phosphorylation in the regulation of RhoGEFs (92). Tyrosine kinase activity was previously detected in *E. histolytica* in response to the activation of β1-integrin-like receptor (41), and specific inhibition of tyrosine protein kinase by genistein resulted in a significant decrease in erythrophagocytosis (8). Moreover, a unique dual-specificity, transmembrane kinase, dubbed PATMK, was recently isolated from *E. histolytica* phagosomes and was shown to participate in erythrophagocytosis and pathogenicity of this parasite (9). While the association of *E. histolytica* tyrosine kinases, particularly PATMK, with RhoGEFs and small GTPases remains to be determined, their important role in phagocytosis is well-supported by recent experimental studies.

Another pathway that has been implicated in regulating the activation of GEFs for Rho/Rac GTPases in mammalian cells is a phosphoinositide 3-kinase (PI3-kinase) signaling pathway (70). PI3-kinases and their lipid products, namely PIP₃ and PI3P, are known to participate in a multitude of cellular processes including cell motility, proliferation, and endocytosis. Studies in mammalian cells
place PI3-kinases upstream of Rac GTPases but downstream of receptor tyrosine kinases (83). The mechanism of Rac GTPase activation by PI3-kinases probably involves the direct binding of RhoGEFs to PIP₃, which is evidenced by the presence of a lipid binding PH domain in all known mammalian GEFs (83). PI3-kinase-catalyzed production of PIP₃ may lead to translocation of RhoGEFs to the plasma membrane and a subsequent increase in a GTP-bound form of Rac GTPases (70). Remarkably, activation of Rac GTPases was shown to activate PI3-kinase resulting in elevated PIP₃ levels (11; 85). Therefore, the functional relationship between PI3-kinase and Rac GTPases may involve a positive feedback loop leading to the amplification of the initial extracellular signal (70).

A putative PI3-kinase has been found in a physical association with phagosomes isolated from *E. histolytica* (58). Moreover, two independent studies demonstrated that inhibition of PI3-kinase by a fungal metabolite, wortmannin, resulted in a significant decrease in erythrophagocytosis in *E. histolytica* (8; 32). These studies were the first to implicate lipids in the regulation of phagocytosis and pathogenicity in *E. histolytica*. More recently, Powell *et al.* demonstrated that PI3P, an important product of PI3-kinase activity, accumulated in forming erythrophagosomal cups of *E. histolytica* (67). This observation of PI3P participation in early phagocytosis is contrary to that reported in mammalian cells, in which PI3P directed the fusion of a formed phagosome with late endosomes (99). Therefore, early involvement of PI3-kinase and its products
during hRBC uptake may represent a unique physiological feature of this parasite
and warrants further investigation.

**Phosphoinositide-based signaling**

**Phosphatidylinositol derivatives as second messenger lipids**

One of the most critical functions of the plasma membrane is to mediate the exchange of information between the cell interior and the extracellular environment. A dynamic rearrangement of plasma membrane proteins ensures a rapid and effective cellular response to extracellular cues. Membrane lipids are emerging as key regulators of protein recruitment to the plasma membrane, their subsequent activation and assembly into larger signaling complexes, as well as their timely inactivation and/or retrieval from the cell surface. Specifically, phosphoinositides (PIs), a minor fraction of membrane lipids, have been implicated in coordinating signaling events at the membrane, possibly by recruiting protein interacting partners that contain appropriate PI recognition domains (69). PIs represent a dynamic pool of membrane-associated lipids, derived from a single parent species, phosphatidylinositol, which is phosphorylated by a variety of kinases at positions 3, 4, and 5 of the inositol ring in seven different combinations (69). Three monophosphates (PI3P, PI4P, and PI5P), three bisphosphates (PI(4,5)P$_2$, PI (3,4)P$_2$, and PI(3,5)P$_2$), and one trisphosphate (PIP$_3$) were identified in the inner leaflet of the plasma membrane and implicated in orchestrating a multitude of signaling cascades. For example,
in mammalian cells, PI4P and P(4,5)P₂ are maintained at high steady state levels in the plasma membrane accounting for approximately 7% of the basal ATP consumption in human platelets (98). P(4,5)P₂ serves as a precursor for inositol-1,4,5-trisphosphate (InsP₃) and diacylglycerol (DAG) synthesis, which act as bona fide second messengers regulating Ca²⁺ release from intracellular compartments and activating protein kinase (PKC), respectively (91). Unlike PI4P and P(4,5)P₂, other PIs are generated only transiently in the plasma membrane or other cellular compartments in response to external stimulation. For instance, a singly phosphorylated phosphatidylinositol derivative, PI3P, is rarely detected in the plasma membrane of unstimulated cells and is commonly found in the association with early and late endosomes, possibly regulating endocytic membrane trafficking, phagocytosis, and autophagy (20; 86). Another signaling phosphoinositide, PIP₃, is transiently generated in the plasma membrane of mammalian cells in response to activation of receptor tyrosine kinases (RTKs) and G-protein signaling (96). Enhanced PIP₃ production has been implicated in a variety of cellular processes in mammalian cells, including cell adhesion (21), polarization and chemotaxis (28), phagocytosis (23; 25), mitogenesis and oncogenesis (69). The rest of this chapter is dedicated to the review of the mechanisms of PIP₃ generation and degradation, its downstream effectors and their lipid binding domains, as well as the role of PIP₃ in various endocytic processes in mammalian cells and in Dictyostelium discoideum, an amoeba closely related to E. histolytica.
**PI3-kinase family: products and inhibitors**

Production of PIP$_3$ in the plasma membrane is mediated by the activity of a PI3-kinase, which is capable of transferring the terminal phosphate of ATP to the hydroxyl group at position 3 of the inositol ring in inositol phospholipids, giving rise to 3’-Pls. Initially, mammalian PI3-kinase was purified as a heterodimer comprising a 110 kDa catalytic subunit (p110$\alpha$) and a 85 kDa regulatory subunit (p85$\alpha$) (90). Subsequently, eight distinct catalytic subunits of PI3-kinase were identified in mammalian cells and were grouped into three functional classes based on the substrate specificity and the structure of their protein domains (27). Class I PI3-kinases includes p110$\alpha$, p110$\beta$, p110$\gamma$, and p110$\delta$, which are primarily found in the cytoplasm in quiescent cells and become recruited to the plasma membrane in response to hormonal stimulation. The preferred substrate for class I PI3-kinases is P(4,5)P$_2$, which is converted to PIP$_3$ upon phosphorylation of its 3’-OH group (27). Class II PI3-kinases represent the least understood group of PI3-kinases, distinguished by their relatively large size (~200 kDa), predominantly membrane localization, and inability to phosphorylate P(4,5)P$_2$ (90). Finally, class III PI3-kinases are represented by a single member, a yeast kinase Vps34, which catalyses the production of PI3P in vivo (27; 90).

The activation of PI3-kinase involves two alternative mechanisms. The regulatory subunit of PI3-kinase, p85$\alpha$, may be recruited to the plasma membrane as a consequence of RTK activation, followed by the mobilization of the catalytic subunit, p110, to the forming signaling complex (15). PI3-kinase may
also become activated by the direct interaction of its catalytic subunit with Ras, a member of the superfamily of small monomeric GTPases (15). A multitude of ligands can activate PI3-kinase signaling pathways including insulin, growth hormones, cytokines, and a variety of chemoattractants resulting in the upregulation of a wide array of signaling cascades. The physiological responses mediated by the PI3-kinase signaling can be grouped into three categories: cell growth/survival, motility, and intracellular vesicle trafficking. The function of PI3-kinase that is most relevant to this study is the regulation of various endocytic processes, namely phagocytosis and pinocytosis, which were analyzed in detail in mammalian macrophages and *D. discoideum* (99; 23; 25; 108) and are described later in this chapter.

Historically, the cellular functions of PI3-kinase were studied using two chemically unrelated PI3-kinase inhibitors: wortmannin and LY294002. Wortmannin is a fungal metabolite, which is known for its potent ability to inhibit PI3-kinase and related cellular functions. Wortmannin is a noncompetitive inhibitor that specifically inhibits PI3-kinase at submicromolar concentrations with an IC$_{50}$ of 2-4 nM and has no effect on PI4-kinase, c-src protein tyrosine kinase, or protein kinase C at these low concentrations (68). However, at higher concentration (~100 fold the concentration required for inhibiting PI3-kinase) wortmannin is also known to inhibit some isoforms of PI4-kinase (61), phospholipase A$_2$ (18), phospholipase D, and a myosin light chain kinase (MLCK) (29). The mechanism of inhibition of PI3-kinase by wortmannin involves
a covalent modification of the Lys-802 residue at the ATP-binding site, which interferes with the catalytic process as well as substrate binding (107).

Another low molecular weight compound that is used to inhibit PI3-kinase is LY294002, a synthetic derivative of the broad-spectrum kinase inhibitor quercetin (90). Unlike wortmannin, LY294002 is a competitive inhibitor with an IC$_{50}$ of 0.5-1.5 µM. This morpholino compound is generally considered to be more specific for PI3-kinases, since at 50 µM it did not inhibit PI4-kinase, DAG-kinase, PKC, PKA, MAPK, S6 kinase, EGFR, and c-src kinases (100). However, recent studies indicate that LY294002 loses its specificity at higher concentrations, inhibiting protein kinases structurally related to PI3-kinases. Importantly, recent studies showed that LY294002 inhibited casein kinase 2 (CK2) at the same concentrations required for the inhibition of PI3-kinase (90). This finding, together with the notion that CK2 is a constitutively active kinase with broad cellular functions, including proliferation and transcription, undermines the utility of this inhibitor for whole-cell assays and must be taken into consideration when interpreting results of experiments with LY294002 (90).

Both wortmannin and LY294002 exhibit little selectivity within the PI3-kinase family, inhibiting all known isoforms. This, unfortunately, prevents the analysis of the involvement of individual PI3-kinase isoforms in a particular cellular process using these reagents (27). Alternative approaches, such as construction of dominant negative mutants, knockout strains, and RNA
interference techniques are currently being employed in different organisms for use in functional studies of PI3-kinase activity.

As mentioned in the previous chapter, data mining of the *E. histolytica* genome revealed a putative PI3-kinase (Genbank Accession No. EAL 44414), which was also found to be physically associated with phagosomes in this parasite using a proteomic analysis (55; 58; 63). This putative kinase is structurally similar to the mammalian class I PI3-kinases as indicated by BLAST sequence alignment. *E. histolytica* PI3-kinase possesses a putative Ras-binding domain (RBD) that has been shown to functionally interact with Ras only in class I PI3-kinases (27). Besides a PI3-kinase catalytic domain, amoebic kinase also exhibits a C2-like domain, which was implicated in lipid binding, and a PIK domain that serves as a scaffold for other domains (27). Based on the functional and structural similarities of *E. histolytica* PI3-kinase with the mammalian class I PI3-kinases, it is likely that this enzyme is responsible for the production of PIP$_3$ and PI3P, which carry out various signaling roles in this parasite.

**PTEN and SHIP phosphatases as regulators of PIs cellular pool**

A striking feature of PIP$_3$ generation in cellular membranes is its spatial and temporal restriction to certain membrane compartments, such as phagocytic cups or leading edges of extending pseudopodia. A local asymmetric rise in PIP$_3$ concentration is essential for proper signaling by this phosphoinositide and appears to be tightly regulated. The mechanism of local restriction of phosphoinositide production is unknown; however, some insight may be gained
from studies of inositol phosphatases, which antagonize PI3-kinase signaling. Two inositol phosphatases are currently known to act as key negative regulators of PIP₃ levels: PTEN (phosphatase and tensin homologue deleted on chromosome 10) and SHIP (SRC Homology 2 domain-containing phosphatases). PTEN phosphatase inactivates the PIP₃ signal by removing the phosphate group from the 3-position of the inositol ring, converting the trisphosphate into PI(4,5)P₂ (54). Unlike PTEN, SHIP is a 5-phosphatase that converts PIP₃ into another signaling phosphoinositide PI(3,4)P₂ (56).

PTEN phosphatase was originally identified as an oncogene in a variety of human cancers and is currently thought to be involved in regulating PI3-kinase-dependent Akt signaling and cell motility (54). Functional studies of PTEN null mutants in *D. discoideum* demonstrated that PTEN-deficient amoebae exhibited a decrease in chemotaxis speed, possibly due to a decrease in cell polarity and a diminished ability to maintain a leading pseudopodum at the front of the cell (30; 103). Moreover, PTEN was found to be excluded from the leading edge of forming pseudopodia in GFP-PTEN expressing cells, but enriched along the lateral sides in plasma membrane. These findings suggest that the presence of PTEN along the lateral sides of plasma membrane eliminates PIP₃ in these areas and thus inhibits the formation of lateral pseudopodia. This leads to a local restriction of PIP₃ accumulation at the leading edge of the cell resulting in a directional movement (30; 103). PTEN activity was also implicated in regulating
phagocytosis in *D. discoideum*, since PTEN null mutants exhibited a significant decrease in their ability to take up yeast cells (25).

A PTEN-like PIP₃ phosphatase was identified in the *E. histolytica* genome (Genbank Accession No. EAL 44414). Interestingly, pull-down assays in *E. histolytica* using GST-EhRab7 and subsequent mass spectrometry revealed that PTEN was physically associated with active EhRab7 (L.A. Temesvari, personal communication). Since EhRab7 has been detected in pre-phagosomal vesicles, it is possible that this small GTPase regulates the delivery of PTEN to sites of phagocytic uptake, where it antagonizes PI3-kinase activity and ensures proper phosphoinositide signaling.

Another inositol phosphatase that may be involved in downregulating plasma membrane PIP₃ levels is SHIP phosphatase. Similar to PTEN, this 5-phosphatase regulates cellular polarization and the formation of the leading edge during the directional movement in chemotaxis (62). Moreover, SHIP was shown to be involved in CR3- and FcγR-mediated phagocytosis in macrophages (16). Currently, there is no evidence that SHIP phosphatase is present in *E. histolytica*. However, a search of the *E. histolytica* genome revealed the existence of four OCRL-like sequences that encode putative inositol-5-phosphatases, which possess a RhoGAP domain and a 5-phosphatase domain capable of dephosphorylating PIP₃ (56). In *D. discoideum*, mutants deficient in OCRL-like phosphatase activity (Dd5P4) were unable to complete phagocytosis of yeast cells due to the persistent presence of PIP₃ at the phagosomal membrane and
failure to close phagocytic cups (56). It is possible that the OCLR-like phosphatases in *E. histolytica* perform a function similar to that of Dd5P4. Further investigations into the role of different inositol phosphatases in *E. histolytica* may elucidate regulatory mechanisms underlying PI-based signaling and virulence in this parasite.

**Proteins with specific lipid-recognizing domains**

*Pleckstrin homology (PH) domains: binding specificity and affinity*

Phosphoinositides carry out their functions as mediators of outside-in cell signaling via the recruitment of downstream proteins with specific lipid-binding domains to the plasma membrane, thus promoting the assembly of interacting partners into larger signaling complexes. Several lipid-recognition domains were identified in a wide variety of phosphoinositide target proteins including pleckstrin homology domains (PH), Phox homology domains (PX), epsin N-terminal homology domains (ENTH), and FYVE-finger domains (Fab1p, YOTB, Vps27p, EEA1). While ENTH and FYVE-finger domains almost exclusively bind to PI(4,5)P2 and PI3P, respectively, PH- and PX- domains may bind different phosphatidylinositol derivatives depending on the proteins from which they are derived (49).

The best characterized protein recognition domain for PIP₃ is a PH domain derived from Bruton’s tyrosine kinase (Btk), a cytoplasmic protein tyrosine kinase of the Tec family that is involved in the maturation of B cells. This PH domain is a small protein module of ~120 amino acids, which exhibits homology to the main
substrate of protein kinase C in platelets, pleckstrin (50). The three-dimensional structure of the PH\textsuperscript{Btk} domain was determined by X-ray crystallography (48). The core of the PH domain consists of a seven-stranded antiparallel β-sheet and a C-terminal α-helix (48). The β1/β2 loop of the PH\textsuperscript{Btk} domain is elongated, and, together with β5/β6 loop, forms a PI-binding pocket, which represents the most positively charged area of the domain (19; 48). The PH\textsuperscript{Btk} domain is known to bind PIP\textsubscript{3} with high specificity and affinity. The first demonstration of this specificity came from studies utilizing a biosensor-based assay and unilamellar liposomes containing different phosphoinositides (80). In this study, a PH\textsuperscript{Btk} domain was expressed as a fusion protein with a glutathione S-transferase (GST) tag, which was immobilized onto a BIAcore sensor chip coated with anti-GST antibody. Subsequently, large liposomes loaded with various phospholipids were injected over the sensor chip surface and refractive indices were measured, indicating that only PIP\textsubscript{3} interacted with the PH\textsuperscript{Btk} domain. Subsequently, another study confirmed the specificity of the PH\textsuperscript{Btk} domain interaction with PIP\textsubscript{3} using water-soluble, \textsuperscript{3}H-labeled PIs and bacterially expressed GST-tagged PH domains captured on beads (71). Moreover, the association of the PH\textsuperscript{Btk} domain with PIP\textsubscript{3} has been demonstrated \textit{in vivo} in NIH3T3 fibroblasts (97). In these cells, a PH\textsuperscript{Btk} domain fused to the N-terminus of GFP localized to the plasma membrane only upon stimulation with EGF or PDGF. This effect was abolished by the addition of wortmannin or LY294002 indicating that GFP-PH\textsuperscript{Btk} translocation to the plasma membrane was dependent on the sustained activity of PI3-kinase(s). Taken
together, these studies demonstrate the utility of the PH\textsuperscript{Btk} domain as a tool to study the subcellular localization of PIP\textsubscript{3} both \textit{in vitro} and \textit{in vivo}.

\textit{PH-domain containing proteins in E. histolytica as putative effectors of PIP\textsubscript{3}}

An \textit{in silico} genome data-mining approach identified 87 proteins with PH lipid-recognition domains in \textit{E. histolytica}. Putative PH-domain containing protein targets of PIP\textsubscript{3} may be grouped into three broad categories: 1) Rho guanine nucleotide exchange factors (RhoGEFs) and GTPase activating proteins (GAPs); 2) Rho/Rac GTPases; 3) protein kinases and phosphatases. A search of the Wellcome Trust Sanger Institute \textit{E. histolytica} genome project revealed that there are approximately 23 Dbl homology (DH) domain-containing GEFs and 6 putative GAPs in \textit{E. histolytica} that possess a PH domain (unpublished data). GEFs act as positive regulators of small GTPases by promoting the formation of active GTP-bound state in these proteins, while GAPs facilitate the hydrolysis of GTP to GDP leading to the attenuation of Rho-GTPase signaling (83). The PH domain of RhoGEFs is invariably located immediately C-terminal to the catalytic DH domain, which suggests a functional interdependence of the two domains (104).

Traditionally, the PH domain was thought to serve as a mere membrane-tethering factor important for RhoGEF translocation to the membrane (104). However, recent studies suggest that the PH domain might perform multiple functional roles in GTPase activation, possibly by cooperating with the DH domain to determine the specificity of GEF interaction with target GTPases and phosphoinositide membrane anchors (76). Several RhoGEFs have been
functionally characterized in *E. histolytica*. For example, a PH-domain-containing *EhGEF1* was shown to preferentially catalyze the exchange of GDP for GTP on the small GTPase, RacG, and affect the reorganization of actin cytoskeleton associated with cell motility (2). Another PH-domain containing RhoGEF, *EhGEF3*, was found to activate *EhRacA* and regulate uroid formation and capping of cell surface receptors in *E. histolytica* (6).

Interestingly, both *EhRacA* and *EhRacG* are implicated in regulating phagocytosis in *E. histolytica* (58). A PH-domain containing *EhRacG* was shown to accumulate around erythrophagosomal vacuoles and was suggested to participate in the contractile activity necessary for phagosomal closure (58). Moreover, overexpression of RacG in *D. discoideum* resulted in an increased rate of phagocytosis, which was abolished in the presence of LY294002 (88). These results demonstrate that PIP$_3$ generation, and subsequent recruitment of RacG to the phagocytic membrane, possibly via the interaction of its PH-domain with PIP$_3$, are required for RacG activation and associated phagocytic activity.

A large number of putative *E. histolytica* protein kinases possess a PH-domain (4; 55). Of the 17 putative p21-activated protein kinases (PAKs) identified in the *E. histolytica* genome, 4 PAKs have a PH-domain positioned N-terminal to the kinase domain (EAL52122, EAL43940, EAL46137, EAL52161) (4). A PH-domain containing *EhPAK2*, a downstream target of *EhRacA*, was shown to play an important role in collagen infiltration, cell movement, and receptor capping in *E. histolytica* (5). Moreover, *EhPAK* (EAL52122) was detected by the proteomic
analysis of isolated phagosomes in *E. histolytica* (58). Unexpectedly, 4 PKA/PKG-like protein kinases, which represent the AGC family of protein kinases in *E. histolytica*, were found to contain a PH domain (4). The presence of a PH domain in *E. histolytica* PKA/PKG-like kinases is unusual because in mammalian systems PH-domains are more common in PKC kinases rather than in members of the AGC family (4). Therefore, it is possible that PKA/PKG-like kinases in *E. histolytica* are capable of translocation from the cytoplasm to the cell membrane in response to appropriate stimulation and represent another putative target of PIP₃. The existence of PH-domain containing PKA/PKG-like kinases is a distinctive feature of *E. histolytica*, and further investigation into the physiological role of these kinases may provide insight into a unique biology of this parasite.

**PIP₃ regulatory and signaling roles in Dictyostelium discoideum and mammalian phagocytes**

The physiological role of PI3-kinases and their products has been studied in detail in mammalian phagocytes and *D. discoideum*. One of the most prominent and well-studied cellular functions of PIP₃ is its participation in directional sensing and cell movement during chemotaxis. PIP₃ is thought to play an integral part in the process of recognizing environmental cues and transmitting this signal across the plasma membrane, leading to the reorganization of the actin cytoskeleton, cell polarization, and movement in the direction of a chemoattractant. The molecular machinery involved in this process is commonly referred to as a cellular compass (28). PIP₃ was recognized as one
of the first components of the cellular compass based on the results of numerous studies that demonstrate its accumulation at the leading edge of moving cells (43; 82). Additional evidence supporting the role of PIP₃ in cell polarization and chemotaxis emerged from the studies of PI3-kinase (PI3K) and SHIP null mutants in *D. discoideum* and neutrophils. For instance, PI3K1 and PI3K2 double knockouts in *D. discoideum* were unable to form functional pseudopodia in the presence of chemoattractants (30). Moreover, PI3Kγ⁻/⁻ neutrophils exhibited a diminished capacity to migrate on albumin-coated surfaces (62). Also, neutrophils lacking SHIP phosphatase and hence incapable of regulating localized PIP₃ production, demonstrated unpolarized morphology (62). While these studies do not exclude the possibility of a parallel signaling pathway that controls directional cell movement, they strongly support a central role of PIP₃ in regulating cell polarization and pseudopodia formation during chemotaxis.

Since PIP₃ is the primary regulator of cytoskeleton dynamics, it is not surprising that this signaling lipid is also important in controlling phagocytosis in mammalian cells as well as in lower eukaryotes. Early studies in macrophages utilizing low molecular weight PI3-kinase inhibitors (wortmannin and LY294002) demonstrated that phagocytosis was impaired in treated cells due to their inability to maximally extend pseudopodia and complete the formation of phagosomes (17). These observations suggested that PI3-kinase inhibition interfered with the process of phagosome formation at or prior to phagosomal closure (17). These conclusions were supported by studies of class I PI3-kinase null mutants, made
by targeted ablation of α and β isoforms of p85, which showed reduced uptake of hRBCs that could not be attributed to the defect in phagosomal maturation (99). A more recent study of PIP₃ subcellular localization during phagocytosis of zymosan particles using a GFP-tagged PH domain of Akt showed that this lipid localized to the phagocytic cup at the moment of closure in neutrophils (23). Similarly, studies of PIP₃ dynamics during phagocytosis in *D. discoideum* using a GFP-PH<sup>GRP1</sup> probe demonstrated that PIP₃-specific fluorescence peaked at the time of phagosomal closure (25). Finally, Zhou and colleagues have demonstrated that PIP₃ was required for membrane ruffling and pseudopodia extension during macropinocytosis in this free-living amoeba (108). Taken together, these results indicate that PIP₃ is important during the early stages of endocytosis in mammalian phagocytes as well as in a lower eukaryote, *D. discoideum*, and does not participate in the processes of phagosomal maturation in these systems.

**Lipid rafts – platforms for PI-based signaling**

A prominent feature of PIP₃ accumulation in specific membrane compartments during chemotaxis and phagocytosis is its localized restriction to certain membrane domains such as phagocytic cups or pseudopodia. Often there is a sharp boundary between the area of PIP₃ enrichment and the rest of the membrane. The mechanism of local PIP₃ restriction is currently unknown. However, it is possible that PIP₃ is restricted to certain membrane domains due to its anchorage to underlying binding partners, mainly proteins. On the other
hand, the structural and physical properties of membranes may allow the restriction of PIP₃ to relevant membrane compartments. Currently, a large body of evidence supports the role of cholesterol-rich raft-like microdomains in coordinating spatial and temporal aspects of PI-based signaling. For instance, a study of PIP₃ localization at phagocytic cups in neutrophils demonstrated that the membrane restriction of PIP₃ could not be completely attributed to its immobilization by anchorage to underlying cytoskeletal elements, and that cholesterol depletion inhibited PIP₃ localization to the phagosomal cups (25). In growing neurites, it has been shown that intact lipid rafts and PIs are required for the recruitment of the Pyk2/Cbl signaling complex upon stimulation with growth factors (36). Thus, it is possible that PIP₃ accumulates within lipid rafts, which serve as spatial and temporal platforms that regulate signal transduction events leading to cytoskeletal remodeling.

The existence of the detergent-resistant, cholesterol-rich membrane domains in *E. histolytica* was previously shown by Laughlin and colleagues (52). Moreover, there is convincing evidence that raft-like membrane microdomains actively participate in virulence-associated signaling events in *E. histolytica*. For instance, it has been shown that an important signaling/adhesion molecule, the Gal/GalNAc-inhibitable lectin, colocalizes with lipid rafts, suggesting that these membrane microdomains may participate in signaling in this parasite (52). Also, raft-like lipids and PI3P were observed to accumulate at the erythrophagocytic cups (B.H.Welter, unpublished data; (67)). Taken together, these results suggest
that PIs may regulate virulence-associated signaling in the context of lipid rafts in *E. histolytica*.

**Summary**

Amoebic dysentery continues to be one of the leading causes of morbidity and mortality among young children in the countries that cannot prevent the fecal-oral spread of the protozoan causative agent, *E. histolytica*. The pathogenesis of invasive intestinal amoebiasis depends on the parasite’s ability to carry out chemotaxis and endocytosis in the host’s bowel lumen, which enable it to colonize the digestive tract and establish the infection. *E. histolytica* trophozoites encounter a variety of host cells and extracellular molecular cues that trigger a series of signal transduction cascades in the pathogen leading to the vesicle trafficking events, cytoskeletal rearrangements, and gene expression that result in augmented virulence of the pathogen. While the role of proteins that regulate virulence-related signaling events in *E. histolytica* has been extensively characterized, regulatory functions of lipids remain largely unknown in this parasite. The goal of this Master’s thesis research project is to examine the role of a second messenger lipid, PIP₃, during endocytosis in *E. histolytica* trophozoites. The specific hypothesis behind the proposed research is that PIP₃, the main product of PI3-kinase activity, regulates signal transduction and vesicle trafficking events essential to various endocytic processes in *E. histolytica* by recruiting downstream signaling proteins with specific
lipid binding recognition domains. The hypothesis is based on the following experimental observations. First, a putative PI3-kinase was identified among the signaling molecules that were physically associated with *E. histolytica* phagosomes (58), and inhibition of PI3-kinase using wortmannin and LY294002 blocked the uptake of hRBCs by this amoeba (8; 32). Second, a singly phosphorylated product of PI3-kinase activity, PI3P, was shown to accumulate in the erythrophagosomal cups and at the sites of bacterial uptake in *E. histolytica* (67). Third, PIP₃ was implicated in coordinating signaling events during endocytosis and chemotaxis in mammalian phagocytes (23) and *D. discoideum* (25). These observations provided the incentive to investigate the distribution of PIP₃ during endocytosis in *E. histolytica*. To this end, we implemented two experimental approaches utilizing GST- and GFP- tagged biosensors to examine both spatial and temporal aspects of PIP₃ distribution during phagocytosis and pinocytosis in *E. histolytica*.

We have shown that PIP₃ accumulated in pseudopodia and erythrophagosomal cups but not in fluid phase pinosomes in *E. histolytica* trophozoites. We also observed high steady-state levels of PIP₃ in the plasma membrane of *E. histolytica* trophozoites, which were not abolished by serum withdrawal. To our knowledge, this is the first report of the temporal and spatial aspects of PIP₃ distribution during phagocytosis in *E. histolytica*. This study also elucidates a unique aspect of the biology of this parasite, namely the stability of PIP₃ in the plasma membrane. This distinguishes the physiology of this parasite...
from that of mammalian host cells. Because endocytosis represents an important
virulence function in this parasite, the present study provides important insight
into the molecular mechanisms of *E. histolytica* pathogenicity.
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CHAPTER 2
PHOSPHATIDYLINOSITOL (3,4,5)-TRISPHOSPHATE IS IMPORTANT IN THE
EXTENSION OF PSEUDOPODIA AND CLOSURE OF
ERYTHROPHAGOSOMES IN ENTAMOEBA HISTOLYTICA

Abstract

Entamoeba histolytica is an intestinal protozoan parasite that causes amoebic dysentery and liver abscess. Phagocytosis is a critical activity for the virulence of this human parasite since it is a prerequisite for tissue invasion and establishment of chronic infection. While the role of many proteins that regulate phagocytosis-related signaling events in E. histolytica has been characterized, the functions of lipids in this cellular process remain largely unknown. In other systems, phosphatidylinositol (3,4,5)-trisphosphate (PIP₃), a major product of PI3-kinase activity, is essential for cell polarization during phagocytosis and chemotaxis. To characterize the spatiotemporal aspects of the subcellular distribution of this lipid in trophozoites during various endocytic processes, we utilized a GFP- and a GST-labeled pleckstrin homology domain derived from mammalian Bruton’s tyrosine kinase, which is known to specifically bind to PIP₃. We have shown that the PIP₃ specific biosensor accumulated at extending pseudopodia and at erythrophagosomal cups in trophozoites exposed to erythrocytes, but did not colocalize with pinocytic compartments during the uptake of a fluid phase marker, dextran. Our results suggest that PIP₃ is involved
during the early stages of pseudopodia formation and phagosomal closure preceding phagocytosis in *E. histolytica*. In addition, we demonstrate that PIP$_3$ exists in high steady-state levels in the plasma membrane of *E. histolytica*, which are not abolished by serum withdrawal. We also provide evidence that PIP$_3$-associated signaling events during phagocytosis may occur in the context of lipid raft membrane microdomains in this protozoan parasite.
Introduction

*Entamoeba histolytica* is an intestinal protozoan parasite that causes amoebic dysentery and liver abscess. A high incidence of *E. histolytica* infection is found in developing countries with low basic hygiene standards and a lack of water sanitation (1; 8; 23). Since *E. histolytica* is mainly a human parasite, improvements in sanitation could help prevent the fecal-oral spread of this pathogen and potentially eradicate the disease. However, overpopulation, tropical climate, and scarce water availability in developing countries, compounded by socioeconomic shortcomings, impede progress in this area and make eradication of amoebiasis unlikely in the foreseeable future (7; 45).

Moreover, since *E. histolytica* is a food and waterborne pathogen with a high potential for mass dissemination, it has been classified as a Category B priority bioterrorism agent. Therefore, there is an elevated need for the development of improved prevention, diagnostic, and treatment methods for dysentery caused by *E. histolytica*. The development of such methods requires a better understanding of the basic biology of this parasite.

*E. histolytica* is contracted by ingestion of its multinucleate infective cysts from fecally contaminated food or water (48). Upon excystation in the small intestine, motile trophozoites are released into the bowel lumen, where bacteria, erythrocytes, and cellular debris serve as a food source. Infection is established when trophozoites adhere to the intestinal wall, penetrate colonic epithelium, and on occasion, disseminate, via the hematogenous route, to extraintestinal sites.
Invasion of the intestinal mucosa by *E. histolytica* depends on the parasite’s ability to destroy host cells in a contact dependent fashion, which is likely mediated via the induction of apoptosis in these targets (25). *E. histolytica*-induced cytotoxicity appears to be intimately linked with phagocytosis, which is emerging as an important virulence factor in this parasite. Specifically, Huston *et al.* have demonstrated that apoptotic cell killing preceded and facilitated phagocytosis of host cells (24). This may serve as a possible mechanism of evasion of the host’s immune system by *E. histolytica*, since engulfment of cellular debris limits the spillage of toxic intracellular components and restricts host inflammatory responses (24). Association of *E. histolytica* with intestinal bacteria, and the subsequent phagocytosis of these bacteria have also been directly linked to the virulence of *E. histolytica* (6; 58). Recent transcriptional profiling of *E. histolytica* exposed to *Escherichia coli* showed that bacteria induced a significant upregulation of protein kinase, ABC transporter, Rho family GTPase, and hsp90 gene expression, which may collectively contribute to the modulation of virulence in this parasite (11). Another phagocytic process that plays an important role in pathogenicity of *E. histolytica* is the uptake of human red blood cells (hRBCs). Clones of *E. histolytica*, unable to take up hRBCs, exhibited a reduction in virulence, which was restored upon the recovery of erythrophagocytosis (35). Moreover, an avirulent *Entamoeba* species, *E. dispar*, exhibits a reduced rate of phagocytosis as compared to virulent species (36). Therefore, understanding the molecular mechanisms of phagocytosis in *E.*
*E. histolytica* may provide insight into factors that contribute to the pathogen’s virulence.

Two recent proteomic screens of isolated *E. histolytica* phagosomes revealed a multitude of proteins that may be involved in the processes of phagosome biogenesis in this parasite (32; 34). A putative phosphoinositide 3-kinase (PI3-kinase) was identified among the signaling molecules physically associated with *E. histolytica* phagosomes. PI3-kinase is known to generate a pool of signaling phosphoinositides (PIs) phosphorylated at the 3’-OH position, namely phosphatidylinositol 3-phosphate (PI3P), phosphatidylinositol (3, 4)-bisphosphate (PI(3,4)P₂), and phosphatidylinositol (3, 4, 5)-trisphosphate (PIP₃) (15). PIs are produced in response to extracellular stimuli and are capable of recruiting a wide variety of protein effectors to the plasma membrane via interaction with their specific lipid-recognition domains. That PI3-kinase may be involved in the regulation of phagocytosis in *E. histolytica* is supported by its localization to phagosomes and the finding that small-molecule inhibitors of PI3-kinase block the uptake of hRBCs by this amoeba (5; 19). These studies are the first to implicate lipids as participants in phagocytosis-associated cell signaling in *E. histolytica*.

A recent study that utilized a FYVE-finger domain that specifically binds to a singly phosphorylated product of PI3-kinase, reported that PI3P accumulated in forming erythrophagosomal cups of *E. histolytica* (38). Interestingly, the putative involvement of PI3P in early phagocytosis is contrary to that reported in
mammalian cells, in which PI3P directs the fusion of a formed phagosome with late endosomes (52). Therefore, early involvement of PI3-kinase during hRBC uptake may represent a unique physiological feature of this parasite that warrants further investigation.

Another product of PI3-kinase activity, PIP₃, has been implicated in coordinating phagocytosis in neutrophils, macrophages and in a soil amoeba, closely related to *E. histolytica, Dictyostelium discoideum* (3; 12; 14; 17; 59). Specifically, studies utilizing GFP-labeled probes containing pleckstrin homology (PH) domains, which specifically bind to PIP₃, demonstrated that PIP₃ localized to phagosomal cups at the moment of phagosomal closure in both mammalian phagocytes and in *D. discoideum* (12; 14).

Currently no data is available on the subcellular localization of PIP₃ in *E. histolytica* during phagosome biogenesis. Importantly, *in silico* genome data-mining approaches revealed that there are approximately 87 proteins encoded by the *E. histolytica* genome that possess a putative PH lipid-binding domain (L.A. Temesvari, unpublished data). Moreover, proteomic analysis of isolated phagosomes in *E. histolytica* detected several PH domain-containing proteins including p21-activated protein kinase, a small GTPase (RacG), and a number of Rho guanine nucleotide exchange factors (RhoGEFs) (32; 34). Interestingly, all of these PH domain-containing proteins are also implicated in the regulation of the actin cytoskeleton and vesicle trafficking, which are central to the process of phagocytosis (2; 29; 32). Therefore, it is possible that PH domain-containing
proteins identified in *E. histolytica* recognize and bind to PIP₃, which leads to their translocation to the plasma membrane and subsequent activation of relevant signaling pathways necessary during phagocytosis.

In this study, we investigated the spatiotemporal aspects of PIP₃ distribution during different endocytic processes in *E. histolytica*. We used GFP- and GST-tagged biosensors containing a PH domain derived from Bruton’s tyrosine kinase (Btk) that specifically binds to PIP₃. We demonstrated that PIP₃ was enriched in pseudopodia and erythrophagosomes but not in fluid phase pinosomes in *E. histolytica* trophozoites. We also observed high steady-state levels of PIP₃ in the plasma membrane of *E. histolytica* trophozoites, which were not abolished by serum withdrawal. Finally, we observed that raft-like membrane microdomains accumulated at the sites of parasite-erythrocyte interaction suggesting that PIs may function in the context of lipid rafts to regulate cytoskeletal reorganization associated with phagocytosis.

**Materials and Methods**

**Strains and culture conditions**

*Entamoeba histolytica* trophozoites (strain HM-1:IMSS) were cultured axenically in TYI-S-33 (13) in glass screw cap tubes at 37°C.

**Generation of GFP and GFP-PH<sup>Btk</sup> expressing *E. histolytica* cell lines**

The GFP-PH<sup>Btk</sup> construct was obtained from the pEGFP-N1 plasmid (SignaGen Laboratories, Gaithersburg, MD) containing the cDNA encoding the
Bruton's tyrosine kinase (Btk) pleckstrin homology (PH) domain cloned between the EcoRI/BamHI sites upstream of eGFP. The GFP-PH\textsuperscript{Btk} construct was amplified from this plasmid by PCR using the following primers containing \( BglII \) and \( SalI \) restriction sites for subsequent cloning into an \( E. histolytica \) expression vector: 5\textquotesingle-CCAGATCTAAATGGCCGCAG-3\textquotesingle and 5\textquotesingle-CCGTCGACTTACTTGTACAGC-3\textquotesingle. The sequence encoding GFP alone used for the generation of the control cell line was also obtained from this plasmid and was amplified using the following primers with \( BglII \) and \( SalI \) restriction sites: 5\textquotesingle-CCGGATCCATGGTGAGCAAGG-3\textquotesingle and 5\textquotesingle-CCGTCGACTTACTTGTACAGCTCGTC-3\textquotesingle (a kind gift of Rhonda R. Powell, Clemson University). The amplified products were subcloned into the pCR 2.1-TOPO plasmid vector (Invitrogen, Carlsbad, CA). Subsequently, constructs were ligated into the \( Entamoeba \) expression vector, pGIR209 ([39]; gift of Dr. W.A. Petri, University of Virginia, Charlottesville, Virginia), which had been digested with \( BglII \) and \( SalI \). This vector confers G418-resistance to host cells and allows for the inducible expression of exogenous proteins via the addition of tetracycline to the medium. It is generally co-transfected with a second vector, pGIR308, that confers hygromycin-resistance and serves as a vehicle for the tetracycline repressor ([39]). Successful construct generation was confirmed by PCR, restriction enzyme analysis, and sequencing.

A log-phase culture of \( E. histolytica \) was stably transfected with the vectors by electroporation as described previously ([53]). A control cell line was
generated by transfecting trophozoites with the expression vector encoding GFP alone. The transfected cultures were selected 24 hours post-transfection and maintained with the addition of 6 µg mL\(^{-1}\) G418 and 15 µg mL\(^{-1}\) hygromycin to the medium. GFP or GFP-PH\(^{\text{Btk}}\) expression was induced by the addition of 5 µg mL\(^{-1}\) tetracycline to the culture medium 24 hours prior to experiments (“induced trophozoites”). As controls, trophozoites that had not been induced to express GFP-PH\(^{\text{Btk}}\) (“un-induced”) were used in some of the experiments. The authenticity of the GFP-tagged protein was verified by Western blot analysis of *E. histolytica* cell lysates as described (55) using 1:1,000 dilution of mouse anti-GFP antibodies (Invitrogen).

**Generation of the GST-PH\(^{\text{Btk}}\) biosensor**

The PH\(^{\text{Btk}}\) was amplified from the pEGFP-N1 plasmid using the following pair of primers: 5’-CCGGATCCTCCAGAAAGAAG-3’ and 5’-CCGAATTCGTTTTAAGCTTCC-3’. The PCR product was subcloned into the pCR 2.1-TOPO plasmid vector (Invitrogen). After digestion with *Bam*H1 and *Eco*R1, the PH domain-encoding DNA fragment was ligated in frame into the polylinker region downstream of the sequence encoding GST in the pGEX-5x-1 expression vector (Amersham Biosciences, Piscataway, NJ). Successful construct generation was confirmed by PCR, restriction enzyme analysis, and sequencing. GST and GST-PH\(^{\text{Btk}}\) fusion proteins were expressed in *Escherichia coli* BL21 (Invitrogen) and purified using glutathione-Sepharose affinity chromatography as previously described (46). The purity of GST and GST fusion
proteins was assessed by SDS-PAGE and silver staining as described (37). Western blot analysis was performed as described (55) to verify the authenticity of GST-tagged proteins using 1:10,000 dilution of anti-GST antibodies (Chemicon, Temecula, CA). The relative concentration of GST or the GST-tagged fusion protein was estimated using spectrophotometry (Biophotometer, Eppendorf, Westbury, NY).

**Microscopy**

For time-lapse video laser scanning confocal microscopy of live trophozoites expressing GFP-PH\textsuperscript{Btk} or GFP alone, cells were incubated on ice for 10 min to release them from the glass surface of the culture tube, pelleted by centrifugation at 500 x g for 5 min at 25°C, and placed in PBS in glass chamber slides for observation using a LSM 510 laser scanning confocal microscope (Carl Zeiss MicroImaging Inc., Thornwood, NY). To observe phagocytosis, GFP-PH\textsuperscript{Btk} expressing cells and control trophozoites were exposed to human red blood cells (hRBCs; U.S. Biologicals, Swampscott, MA) (ratio of \textit{E. histolytica}:hRBCs = 1:100) during the imaging session. Time-lapse images were collected at 5-second intervals. The moment of trophozoite adhesion to the erythrocyte was designated as t\textsubscript{0}. Image analysis was carried out via X,Y,Z plots generated with Image J software (U.S. National Institute of Health, Bethesda, MD).

For immunofluorescence microscopy, \textit{E. histolytica} trophozoites were incubated with hRBCs (ratio of \textit{E. histolytica}:hRBCs = 1:100) for 1 min, green-fluorescent protein (GFP)-labeled \textit{E. coli} (Bio-Rad Laboratories, Hercules, CA)
for 15 min, or lysine-fixable tetramethylrhodamine isothiocyanate (TRITC)-
dextran (0.3 mg/ml) (10 kDa, Invitrogen) for 2, 10, 30, and 60 min. Cells were
subsequently, fixed, permeabilized, and stained with GST or GST-PH\textsuperscript{Btk} as
described (38). Trophozoites stained with GST alone served as control for these
experiments. Alexa Fluor 488-conjugated rabbit anti-GST antibody (1:2000)
(Invitrogen) or Texas Red-conjugated goat anti-GST antibody (1:1000) (Rockland
Immunochemicals, Gilbertsville, PA) were used for GST-PH\textsuperscript{Btk} immunodetection.
Stained trophozoites were mounted in the glycerol/PBS (1:1) solution on the
microscope slides and observed using a Zeiss LSM 510 confocal microscope. To
confirm the authenticity of GST-PH\textsuperscript{Btk} staining, cells were treated with a range of
concentrations (60, 100, 250, and 500 nM) of the PI3-kinase inhibitor,
wortmannin (Sigma Chemicals, St. Louis, MO) for 15 or 30 min prior to fixation
and decoration with the GST-PH\textsuperscript{Btk} biosensor. Cell viability, after wortmannin
treatment, was estimated using Trypan Blue exclusion (0.5 mg mL\textsuperscript{-1}).

To visualize lipid rafts, cells were stained with the fluorescent lipid
analogue DiIC\textsubscript{16}, 1.1 µM, (Invitrogen) for 2 min at room temperature prior to
fixation as previously described (30). Stained trophozoites were observed by
confocal microscopy as described (57). The raft image was kindly provided by
Brenda H. Welter.

In some instances, probe-specific fluorescence was measured and
quantified using LSM 5.1 Image Browser software (Carl Zeiss) and fluorescence
values were corrected for cell surface area.
**Measurement of erythrophagocytosis and fluid phase pinocytosis**

Measurements of phagocytosis of hRBCs in *E. histolytica* trophozoites expressing GFP-PH\textsuperscript{Btk} construct were carried out according to the methods of Voigt *et al.* (54). Fluid phase pinocytosis was measured using the fluorescent fluid phase marker, FITC-dextran (10 kDa; 2 mg mL\textsuperscript{-1}) (Invitrogen) as previously described (56). Trophozoites expressing GFP alone and “un-induced” transfectants were used as controls in these experiments.

**Statistical analyses**

All values are given as the mean±SD. Statistical analyses were performed using GraphPad Instat® V.3 with one-way ANOVA and a Tukey-Kramer Multiple Comparison Test. *P* values less than 0.001 were considered extremely significant (***) and *P* values less than 0.01 were considered highly significant (**).

**Results**

**PIP\textsubscript{3} is localized to pseudopodia and erythrophagosomes in *Entamoeba histolytica***

While the role of proteins that regulate endocytosis in *Entamoeba histolytica* has been extensively characterized, the regulatory functions of lipids remain largely unknown in this parasite. An important derivative of phosphatidylinositol, PIP\textsubscript{3}, has been implicated in coordinating signaling events during phagocytosis and macropinocytosis in *Dictyostelium discoideum* and
mammalian phagocytes (12; 14). Presently, no data is available about the localization of PIP₃ during phagocytosis and pinocytosis in *E. histolytica*.

In order to examine the spatial and temporal aspects of PIP₃ distribution in *E. histolytica* during various endocytic processes, *E. histolytica* trophozoites were stably transfected with a GFP-tagged PH domain from Bruton’s tyrosine kinase (Btk). PH<sup>Btk</sup> domains, fused to GFP or GST tags, have been previously shown to bind to PIP₃ with high affinity and specificity both *in vitro* and *in vivo* (26; 40; 44; 51). Stable cell lines were generated by selection with hygromycin and G418. The authenticity of expression was established by Western blotting of cell lysates prepared from the transfected trophozoites with anti-GFP antibody. Western blot analysis revealed a unique band of 46 kDa, which is equivalent to the combined molecular weight of the PH<sup>Btk</sup> domain and a GFP tag (Fig. 2.1, lane 3).

![Western blot analysis of cell lysates prepared from wild-type cells or transformed cells before (un-induced trophozoites) and after (induced trophozoites) induction of GFP-PH<sup>Btk</sup> expression with tetracycline. Cell lysates were analyzed on a 12% SDS-polyacrylamide gel, electroblotted onto polyvinylidene difluoride membrane and probed with polyclonal anti-GFP antibody.](image)

**Fig. 2.1.** Western blot analysis of cell lysates prepared from wild-type cells or transformed cells before (un-induced trophozoites) and after (induced trophozoites) induction of GFP-PH<sup>Btk</sup> expression with tetracycline. Cell lysates were analyzed on a 12% SDS-polyacrylamide gel, electroblotted onto polyvinylidene difluoride membrane and probed with polyclonal anti-GFP antibody.

The dynamic distribution of PIP₃ in live *E. histolytica* trophozoites expressing GFP-PH<sup>Btk</sup> was assessed by time-lapse video fluorescence microscopy. Observation of randomly moving trophozoites revealed a prominent
localization of the GFP-PH$_{Btk}$ probe in extending pseudopodia (Fig. 2.2C, arrows). In contrast, control cells expressing GFP protein alone exhibited cytosolic fluorescence, which was excluded from forming pseudopodia (Fig. 2.2B and E, arrows). Localization of PIP$_3$ to extending pseudopodia in *E. histolytica* is consistent with the well-established roles of PI3-kinase and PIP$_3$ in controlling the generation of pseudopodia and cell movement in other professional phagocytes (27).

To gain insight into the role of PIP$_3$ in phagocytosis of hRBCs, *E. histolytica* trophozoites expressing GFP-PH$_{Btk}$ were challenged with human...
erythrocytes and visualized by time-lapse fluorescence imaging. The ingestion of a single erythrocyte occurred over a 60-80 second time interval, as evidenced by the observation of three independent phagocytic events. Each phagocytic event began with trophozoite binding to the erythrocyte followed by the extension of pseudopodia around the particle, phagosomal closure, and subsequent internalization of the newly sealed phagosome (Fig. 2.3, A and B). No localized accumulation of the GFP-PH\textsuperscript{Btk} probe was detected at the site of erythrocyte attachment, suggesting that PIP\textsubscript{3} is not involved in the early stages of particle binding and adhesion (Fig. 2.3A, t\textsubscript{0}). However, a peak in PIP\textsubscript{3} fluorescence intensity was detected upon phagosomal closure and was accompanied by a decrease in GFP-PH\textsuperscript{Btk} intensity in the bulk membrane (Fig. 2.3A, t\textsubscript{40}). The asymmetric rise in PIP\textsubscript{3}–specific signal at the cup and concomitant drop in the membrane fluorescence was analyzed using 3D plots generated from corresponding single-plane confocal images, in which fluorescence intensity is represented as the z-axis of X,Y,Z plots (Fig. 2.3B, t\textsubscript{40}). This accumulation was transient, lasting less than 10s, and was followed by a rapid dissipation of the signal during the internalization of the phagosome (Fig. 2.3, t\textsubscript{50}-t\textsubscript{80}). This observation is consistent with previously described PIP\textsubscript{3} dynamics during the phagocytosis of zymosan particles in neutrophils and \textit{D. discoideum} (12; 14). PIP\textsubscript{3} specific fluorescence was never observed in intracellular pinosomes, which are used for liquid uptake (Fig. 2.2C and Fig. 2.3). Taken together, these results
indicated that PIP₃ was present in the pseudopodia, as well as in the erythrophagosomes, in *E. histolytica*.

**Fig. 2.3.** Time-lapse confocal images of GFP-PH²tk - expressing *E. histolytica* trophozoites during the phagocytosis of hRBCs. (A) Transformed *E. histolytica* cells were exposed to hRBCs and images were collected in a single confocal plane at the indicated time points. Adhesion of *Entamoeba* to the erythrocyte was defined as time zero and subsequent images describe the uptake of a single particle: extension of pseudopodia around the erythrocyte (t₃₀), closure of the erythrophagosome (t₄₀), and internalization of the newly sealed phagosome (t₅₀-t₈₀). Shown time-lapse series is indicative of at least three independent phagocytic events. Both fluorescence images as well as fluorescence images merged with DIC images are shown. (B) Single confocal plane images captured by time-lapse microscopy are presented as a corresponding series of X, Y, Z plots with the z axis representing GFP-PH²tk --specific fluorescence. Distribution of GFP-PH²tk fluorescence follows the formation of the erythrophagocytic cup and peaks at the time of phagosomal closure at 40 s. Scale bars represent 10 µm.
Expression of GFP-PH\textsuperscript{Btk} probe caused a decrease in erythrophagocytosis, but had no effect on pinocytosis

Microscopic comparison of erythrophagocytosis in GFP-PH\textsuperscript{Btk} expressing trophozoites and wild-type or GFP control cells indicated that phagocytic events were rare in the cells expressing the GFP-PH\textsuperscript{Btk} probe. This suggested a functional defect in phagocytosis prompting us to quantitate phagocytosis in \textit{E. histolytica} cells expressing the GFP-PH\textsuperscript{Btk} probe.

Substantial evidence indicates that overexpression of PH-containing proteins may interfere with PI-based signaling and impair certain cellular functions, presumably by sequestration of endogenous lipids and/or their effector proteins (9; 50). To test whether expression of the GFP-PH\textsuperscript{Btk} biosensor in intact \textit{E. histolytica} cells interfered with their ability to take up hRBCs, trophozoites were exposed to human erythrocytes. After 10 min of exposure, extracellular erythrocytes were lysed hypotonically with distilled water and the level of ingested heme was measured by spectrophotometry. Erythrophagocytosis in GFP-PH\textsuperscript{Btk} expressing trophozoites was inhibited by 78% or 69% as compared to the wild-type or GFP control cells, respectively (Fig. 2.4 A). To assess the overall cell survival and proliferation in GFP-PH\textsuperscript{Btk} expressing \textit{E. histolytica}, doubling time and viability assays were performed. The expression of the PIP\textsubscript{3} biosensor did not affect cell survival and proliferation in \textit{E. histolytica} trophozoites, suggesting that the observed phagocytic defect was not due to a general deleterious effect of probe expression in the cells (data not shown).
Remarkably, pinocytosis of a 10 kDa fluid-phase marker, FITC-dextran, was not affected by the expression of the GFP-PH$^{Btk}$ (Fig. 2.4B). This observation is consistent with microscopic data showing that the PIP$_3$ probe did not localize to the intracellular pinocytic compartments. The lack of pinocytosis inhibition in GFP-PH$^{Btk}$ expressing trophozoites further verifies the selective inhibitory effect of the biosensor expression on phagocytosis in *E. histolytica*. 
Fig. 2.4. Expression of the GFP-PH$_{Btk}^{\mathrm{Bik}}$ biosensor inhibited erythrophagocytosis in *E. histolytica*, but did not affect fluid phase pinocytosis. (A) Quantification of erythrophagocytosis. *E. histolytica* trophozoites were challenged with hRBCs (*Entamoeba*:erythrocyte ratio; 1:100) for 10 min, lysed and analyzed for the ingested heme concentration using spectrophotometry. The data are reported as absorbance at 405 nm (A 405) as well as the fraction (%) of heme absorbance in wild-type cells (absorbance in wild-type cells was arbitrarily set to 100%). A highly significant decrease in erythrophagocytosis (**, P<0.01) was observed for GFP-PH$_{Btk}^{\mathrm{Bik}}$-expressing trophozoites as compared to wild-type cells. (B) Quantification of fluid phase pinocytosis. *E. histolytica* cells was incubated in serum-free medium supplemented with the fluorescent fluid phase marker, FITC-dextran (10 kDa, 2 mg mL$^{-1}$), for 30 min and intracellular fluorescence was measured by spectrofluorimetry using excitation and emission wavelengths of 485 and 528 nm, respectively. The data are reported as fluorescence values as well as the fraction (%) of fluorescence detected in wild-type cells (fluorescence in wild-type cells was arbitrarily set to 100%). The data represent the mean±SD of 3 independent experiments.
In addition to significantly inhibited erythrophagocytosis, GFP-PH\textsuperscript{Btk} expressing trophozoites exhibited a striking difference in the appearance of their cytoplasm when compared with the wild-type control cells using differential interference contrast (DIC) imaging (Fig. 2.2, D and F). While wild-type cells exhibited a highly vacuolated cytoplasm, presumably due to the formation of phagosomes and macropinosomes, the dimensions of vacuoles in GFP-PH\textsuperscript{Btk} expressing cells were remarkably smaller, possibly corresponding to the pinosomal compartments. Taken together, these results indicate that expression of the GFP-PH\textsuperscript{Btk} probe inhibited the phagocytosis of hRBCs, while having no effect on the uptake of the fluid-phase marker, suggesting that erythrophagocytosis and pinocytosis may employ different molecular mechanisms.

*E. histolytica* exhibits high steady state levels of PIP\textsubscript{3} in plasma membrane, which are inhibited by wortmannin in a dose-dependent manner but are not abolished by serum starvation: a GST-PH\textsuperscript{Btk} biosensor approach

Since expression of the GFP-PH\textsuperscript{Btk} construct in *E. histolytica* trophozoites inhibited erythrophagocytosis, we decided to use a complementary experimental approach to localize PIP\textsubscript{3} using a glutathione-S-transferase (GST) -tagged PH\textsuperscript{Btk} containing biosensor. Although the experimental approach utilizing GST-PH\textsuperscript{Btk} excludes the temporal aspects of PIP\textsubscript{3} distribution, since trophozoites must be fixed prior to decoration with the GST-tagged probe, it provides a practical
alternative to investigate the spatial aspects of PIP₃ distribution without disrupting relevant cellular functions.

Bacterial expression of GST (control) and GST-PHᵦtk fusion proteins and subsequent affinity purification yielded a 49 kDa chimeric protein (Fig. 2.5 A, lane 3), which is equivalent to the combined molecular weight of the GST tag (Fig. 2.5 A, lanes 1 and 2) and the PHᵦtk domain. The authenticity of the GST and GST-PHᵦtk recombinant proteins was verified by Western blot analysis using anti-GST antibodies (Fig. 2.5 B).

![Fig. 2.5. Construction and purification of GST and the GST-PHᵦtk fusion protein. (A) SDS-polyacrylamide gel and silver stain of GST (lanes 1 and 2) and GST-PHᵦtk (lane 3) protein preparations after affinity purification. (B) Western blot analysis using anti-GST antibody of purified GST (lane 1 and 2) and GST-PHᵦtk (lane 3) verifying the authenticity of GST-tagged chimera.](image)

To test the utility of the generated biosensor, unstimulated *E. histolytica* trophozoites were fixed, permeabilized, and stained with GST-PHᵦtk and GST control protein as previously described (38). The GST tag was detected using Alexa Fluor 488 (green)-labeled rabbit anti-GST antibody. Imaging revealed that in the majority of fixed trophozoites the GST-PHᵦtk biosensor uniformly decorated plasma membranes producing a high-intensity signal along the cell periphery, giving it a ring-like appearance (Fig. 2.6 Aa). Control cells stained with the GST
protein lacking a PH\textsuperscript{Btk} domain did not exhibit any fluorescence (Fig. 2.6 Ab). This observation supports the authenticity of the staining pattern observed with the GST-PH\textsuperscript{Btk} biosensor.

To further verify the specificity of the biosensor interaction with PIP\textsubscript{3}, we utilized a known inhibitor of PI 3-kinase, wortmannin. It was previously reported that the IC\textsubscript{50} of wortmannin for erythrophagocytosis in \textit{E. histolytica} was approximately 40 nM (38). Since binding of a PI3P-specific probe was maximally inhibited by 60 nM wortmannin in a previous study (38), this concentration was used in our experiments as a starting point to test the response of the PIP\textsubscript{3} specific probe to PI 3-kinase inhibition. Treatment with 60 nM wortmannin did not have an effect on probe-specific membrane staining in \textit{E. histolytica} trophozoites (data not shown). To test the effect of higher wortmannin concentrations on GST-PH\textsuperscript{Btk} staining, \textit{E. histolytica} cells were treated with 100 nM, 250 nM, and 500 nM wortmannin for 15 or 30 min prior to fixation and decoration with the biosensor, and the intensity of the probe-specific membrane fluorescence was measured. The intensity values were then normalized for the cell surface area. By this measure, wortmannin inhibited GST-PH\textsuperscript{Btk} membrane staining in \textit{E. histolytica} trophozoites in a dose- and time-dependent manner (Fig. 2.6 B and C). The viability of trophozoites exposed to the wortmannin concentrations specifically used in this experiment was not affected, since a Trypan Blue exclusion assay showed that 96.7% cells remained viable after the treatment with 500 nM wortmannin for 30 min (data not shown). These data indicate that the GST-PH\textsuperscript{Btk}
membrane staining observed in *E. histolytica* is dependent on the phosphorylated products of PI 3-kinase and lends support to the specificity of the biosensor interaction with PIP₃.

**Fig. 2.6.** Wortmannin inhibited GST-PH₅k plasma membrane staining in a dose- and time-dependent manner, indicating specificity of the biosensor interaction with PIP₃. (A) Immunofluorescence microscopy using the GST-PH₅k biosensor. *E. histolytica* trophozoites were fixed, permeabilized and decorated with GST-PH₅k probe followed by Alexa Fluor 488-conjugated goat anti-GST secondary antibody (green). GST-PH₅k uniformly decorated plasma membranes giving rise to a ringed appearance of the cell (a). As controls, cells were probed with GST protein, which did not exhibit any staining (b). Corresponding DIC images are shown (c and d). (B) Treatment with a range of concentrations of wortmannin for 15 and 30 min inhibited GST-PH₅k plasma membrane staining in a time- and dose-dependent manner. Scale bars represent 10 µm. (C) Quantification of the effect of wortmannin treatment on GST-PH₅k plasma membrane staining. Single confocal plane images of pre-treated, fixed and decorated trophozoites were captured and membrane fluorescence intensities normalized for cell surface area were measured using a Zeiss LSM image processing software. Treatment with 500 nM wortmannin for 30 min completely abolished the PIP₃-specific membrane fluorescence (***, P<0.001, as compared to un-treated cells). The data represent the mean±SD of 40 cells per treatment.
Interestingly, a 15-minute treatment with 60 nM wortmannin has been previously shown to eliminate PI3P staining in this parasite (38), whereas a 30-minute treatment with 500 nM wortmannin was required to abolish PIP₃ staining in these experiments. Together with the microscopic observation of high basal levels of PIP₃ in unstimulated *E. histolytica*, these findings suggest that PIP₃ is constitutively produced and/or maintained at high steady-state levels in the plasma membrane of *E. histolytica* grown in axenic culture media.

It is well established that in quiescent mammalian cells PIP₃ levels are virtually undetectable, and transient generation of this signaling lipid is achieved only by stimulation of cells with growth factors and chemokines (22). It is possible that *E. histolytica* trophozoites grown in culture medium, supplemented with fetal bovine serum may be exposed to growth factors and chemoattractants that constitutively activate PIP₃ production in their cell membranes. To test this hypothesis, *E. histolytica* were cultured in a serum-free medium for 14 hours and then fixed, stained with the GST-PH²⁷Btk probe and visualized using fluorescence confocal microscopy. Quantitative measurement of membrane fluorescence in trophozoites stained with the probe indicated that serum starvation did not abolish GST-PH²⁷Btk staining in the plasma membrane of *E. histolytica* (Fig. 2.7). Similarly, probe-specific staining was not altered by a one-hour incubation of trophozoites in a phosphate buffer (PBS) (data not shown). Therefore, in contrast to mammalian cells, where PIP₃ is transiently generated in response to
stimulation with growth factors and chemoattractants, *E. histolytica* exhibits high steady-state levels of PIP$_3$.

**Fig. 2.7.** Serum starvation did not abolish GST-PH$^{Btk}$ staining in the plasma membrane of *E. histolytica*. (A) Immunofluorescence microscopy of *E. histolytica* grown in complete medium and incubated in the serum-free medium for 14 hrs using the GST-PH$^{Btk}$ biosensor and Alexa Fluor 488-conjugated goat anti-GST secondary antibody (green). (B) Quantification of the effect of serum-withdrawal on GST-PH$^{Btk}$–specific membrane fluorescence. Single confocal plane images of pre-treated, fixed and decorated trophozoites were captured and membrane fluorescence intensities normalized for cell surface area were measured using a Zeiss LSM image processing software. The data represent the mean±SD of 40 cells per treatment.
A GST-tagged PIP$_3$ specific biosensor accumulated at the erythrophagosomal cups, but not in fluid-filled pinosomes in *E. histolytica*

To confirm the subcellular localization of PIP$_3$ during phagocytosis using a GST-PH$^{Btk}$ biosensor, *E. histolytica* trophozoites were exposed to hRBCs for 1 minute. Subsequently, the cells were fixed, permeabilized and stained with GST-PH$^{Btk}$ or with GST control protein as previously described (38). The GST tag was detected using Texas red–labeled goat anti-GST antibody. Different stages of erythrocyte uptake were captured using laser-scanning confocal microscopy in different trophozoites. Consistent with our experiments on live GFP-PH$^{Btk}$ expressing *E. histolytica*, the GST-PH$^{Btk}$ biosensor labeled erythrophagocytic cups at the point of the phagosome closure. Specifically, GST-PH$^{Btk}$ staining was observed in the pseudopodia extending around a partially engulfed erythrocyte (Fig. 2.8A). The PIP$_3$-specific probe was also detected in presumably newly sealed phagosomal membranes (Fig. 2.8B). However, no probe-specific staining was observed in the internalized erythrophagosomes located just beneath the plasma membrane (Fig. 2.8C), which further supports our conclusion that PIP$_3$ rapidly dissipates from the phagosomal membrane after its closure is complete. Single confocal plane images indicated that local enrichment in GST-PH$^{Btk}$ staining around the erythrophagosomal cup was accompanied by a dramatic decrease in basal PIP$_3$ levels throughout the membrane (Fig. 2.8, A and B). Upon phagosomal closure, however, the probe-specific staining was restored in the cell membrane to its originally high basal levels (Fig. 2.8C). Importantly, erythrocyte
membranes were not stained with the GST-PH$_{Btk}$ probe as evidenced by the extracellular hRBCs captured in the same confocal plane as the labeled $E. histolytica$ cell (Fig. 2.8B), demonstrating that GST-PH$_{Btk}$ staining is specific for the $E. histolytica$ membrane. Control cells that were stained with the GST protein alone did not exhibit any fluorescence at the erythrophagosomal cups (Fig. 2.8D). As expected, wortmannin treatment (500 nM, 30 min) prevented the uptake of hRBCs and abolished GST-PH$_{Btk}$ staining at the cup (Fig. 2.8E). Consistent with the previous report in $E. histolytica$ (38), wortmannin did not impair adhesion of the trophozoites to hRBC as indicated by numerous erythrocytes attached to the trophozoites' surface and partially engulfed hRBCs by arrested pseudopodia. Together, these results confirm that PIP$_3$ is localized to phagosomes at the point of closure of the cups and does not seem to participate in the early stages (adhesion to hRBCs) or later stages (maturation) of phagocytosis in $E. histolytica$. 
Fig. 2.8. GST-PH\textsuperscript{Btk} probe decorated extending pseudopodia and forming erythrophagosomal cups in \textit{E. histolytica}. Trophozoites were challenged with hRBCs for 1 min and subsequently fixed, permeabilized and stained with GST-PH\textsuperscript{Btk} probe. GST tag was visualized using Texas Red-conjugated goat anti-GST antibody (red). PIP\textsubscript{3}–specific fluorescence is observed in the pseudopodia extending around the erythrocyte (A) and in the presumably newly sealed phagosomes (B). That increase in GST-PH\textsuperscript{Btk} staining at the phagosomal cup is associated with the drop in fluorescence in the bulk plasma membrane is clearly seen in A and B. Upon phagosomal internalization, PIP\textsubscript{3}–specific membrane staining was restored in the membrane (C). The PIP\textsubscript{3} signal was undetectable in the control cells stained with GST protein lacking the PH domain (D). Pre-treatment with wortmannin (500 nM; 30min) abolished GST-PH\textsuperscript{Btk} staining of erythrophagosomes and arrested the extension of the pseudopodia around the erythrocyte (E). Corresponding DIC images are shown (middle panels). Scale bars represent 10 \(\mu\text{m}\).

Since expression of the GFP-PH\textsuperscript{Btk} probe in \textit{E. histolytica} cells did not appear to affect the uptake of a small fluid phase marker, FITC-dextran, and previous work has shown that PI3P did not co-localize with pinosomes harboring dextran (38), we hypothesized that pinocytosis in \textit{E. histolytica} is not dependent on at least two products of PI3-kinase activity, PI3P and PIP\textsubscript{3}. To further test the
involvement of PIP₃ in pinocytosis, *E. histolytica* trophozoites were exposed to lysine-fixable tetramethylrhodamine isothiocyanate (TRITC)-dextran (10 kDa) for 2, 5, 10, and 60 minutes prior to fixation and staining with GST-PH<sup>ßtk</sup>. The probe was visualized using Alexa Fluor 488 (green)-labeled rabbit anti-GST antibody. Similar to a previous report on PI3P, the PIP₃-specific probe did not co-localize with fluid phase pinosomes at any time (uptake of dextran at 60 min is shown in Fig. 2.9). These data are consistent with our observation that expression of GFP-PH<sup>ßtk</sup> did not inhibit the uptake of 10 kDa dextran and indicate that PIP₃ may not be involved in the pinosomal trafficking in *E. histolytica*. Several previous reports suggested that the molecular mechanisms of particle uptake might vary depending on their size (3; 38). Our observations that PIP₃ is required for erythrophagocytosis, but not for pinocytosis, are consistent with the model of size-selective endocytosis.
Lipid raft membranes accumulated at sites of interaction of *E. histolytica* with human red blood cells

Using a GST-PH\textsuperscript{Btk} biosensor, we demonstrated that PIP\textsubscript{3} accumulated at the erythrophagosomal cups in *E. histolytica* and that its distribution was restricted to the site of hRBCs uptake forming a distinct boundary between the cup and the surrounding membrane. Previous studies of PIP\textsubscript{3} localization at phagocytic cups in neutrophils demonstrated that the membrane restriction of...
PIP₃ could not be attributed to its immobilization by anchorage to the underlying cytoskeletal elements, and that cholesterol depletion completely inhibited PIP₃ localization to the phagosomal cups (12). These observations suggest that cholesterol-rich membrane microdomains may play a role in PIP₃ restriction to the phagocytic cups. The existence of detergent-resistant, cholesterol-rich membrane domains, i.e. lipid rafts, in *E. histolytica* was previously demonstrated (30). To determine if lipid rafts are involved in the phagocytic uptake of erythrocytes, we utilized DilC₁₆, a lipid probe sensing ordered membrane phases, to stain *E. histolytica* exposed to hRBCs. Fluorescence imaging indicated that DilC₁₆-stained raft-like microdomains accumulated at the sites of parasites’ interaction with hRBCs (Fig. 2.10, A and B). Since these trophozoites were stained with DilC₁₆ prior to exposure to the erythrocytes, we are confident that the accumulation of red fluorescence is not due to staining of the hRBC membrane. In support of this, a second optical section of the same field of cells is shown in which more of the hRBCs are in the plane of focus and are not, themselves, stained (Fig. 2.10, C and D). Our observation that lipid rafts accumulate at the sites of erythrocyte uptake indicates that these membrane microdomains may participate in the regulation of phagocytosis of hRBCs in *E. histolytica*. In view of recent evidence that lipid rafts function as physical and temporal platforms for phosphoinositide-based signaling in mammalian cells (12, 21), it is tempting to speculate that lipid rafts may precede and facilitate localized accumulation/synthesis of signaling phosphoinositides in the phagocytic
membranes in *E. histolytica*. While our results suggest lipid rafts involvement during erythrocyte uptake in *E. histolytica*, the functional link between lipid rafts and phosphoinositol signaling during erythrophagocytosis in *E. histolytica* remains to be determined.

**Fig. 2.10.** Lipid raft specific stain accumulated at the sites of *Entamoeba*:erythrocyte interaction. *E. histolytica* cells were stained with DilC<sub>16</sub> to label lipid rafts and then exposed to hRBCs. Raft membranes accumulated at the erythrophagosomal cups (A,B). Panels C and D represent a different optical section of the field in panels A and B to demonstrate that the hRBCs are not stained with DilC<sub>16</sub>. Both fluorescence images as well as fluorescence images merged with DIC images are shown. Scale bars represent 10 µm.

*PIP<sub>3</sub>* is enriched at the leading edge of pseudopodia associated with bacterial uptake in *E. histolytica*

Testing the involvement of PIP<sub>3</sub> in the uptake of a particle other then RBCs or fluid-phase markers could possibly reveal yet another pattern of PIP<sub>3</sub> distribution. *E. histolytica* survival in the large intestine of its host depends on its ability to ingest not only hRBCs and cellular debris but also bacteria, which may contribute to the pathogen’s virulence (6; 11; 58). Moreover, PI3P was previously shown to accumulate at the sites of bacterial uptake in *E. histolytica*, and
wortmannin was reported to inhibit the phagocytosis of microorganisms by this parasite (19). These observations prompted us to extend our investigations to explore the distribution of PIP₃ in phagocytosis of GFP-expressing *E.coli*. To investigate the cellular localization of PIP₃ during phagocytosis of bacteria, *E. histolytica* trophozoites were challenged with GFP expressing *E. coli* for 10 or 15 min and then, fixed and stained with the GST-PHᵦtk biosensor. Anti-GST secondary antibody was used to detect the reporter. Immunofluorescence microscopy showed that the GST-PHᵦtk biosensor was strongly enriched at the leading edge of pseudopodia extending around a group of GFP-*E.coli* (Fig. 2.11) and did not co-localize with already internalized bacteria (Fig. 2.11, C and D, arrows). This finding suggests that PIP₃ may function during early stages of phagocytosis of bacteria, which involves the initiation and extension of pseudopodia in the direction of these particles. This finding lends further support to our previous observation of PIP₃ involvement in the processes of pseudopodia formation.

Together with our observations utilizing a GFP-PHᵦtk biosensor or wortmannin, these results demonstrate that PIP₃ is likely to be involved in the formation of pseudopodia during both cell movement and particle uptake as well as in the closure of erythrophagosomes in *E. histolytica*. On the contrary, pinocytosis of a small fluid-phase marker in this parasite does not seem to be dependent on either PIP₃ or PI3P as demonstrated here and elsewhere (38).
Discussion

In the present study we utilized GST- and GFP-tagged biosensors, consisting of a PH domain that specifically binds to PIP$_3$, to examine the subcellular localization of this important signaling lipid in *E. histolytica* during various endocytic processes. We have shown that PIP$_3$-specific biosensors accumulated at the extending pseudopodia in *E. histolytica* during both random movement and uptake of bacteria. Additionally, PIP$_3$ localized to the erythrophagosomal cups at the point of phagosomal closure, which coincided with a drop in fluorescence in the surrounding membrane. Moreover, we
demonstrated that PIP\(_3\) may not be involved in pinocytosis in this parasite, since PIP\(_3\)–specific probes did not co-localize with pinocytic compartments. Finally, we have shown that raft-like microdomains accumulated at the sites of parasite interaction with hRBCs thus providing a possible explanation for the spatial restriction of PIP\(_3\) and PI3P at the phagosomal cups.

In order to characterize both spatial and temporal aspects of PIP\(_3\) accumulation in live trophozoites, we created an *E. histolytica* cell line stably expressing a GFP-tagged PH\(^{Btk}\) domain. However, we observed that expression of GFP-PH\(^{Btk}\) inhibited phagocytosis of hRBCs in *E. histolytica*. The inhibitory effect of expressed PH domains on selective cellular functions is not unprecedented. Varnai *et al.* have previously demonstrated that expression of a GFP-PH\(^{Btk}\) construct in fibroblasts inhibited activation of one of the most well known downstream targets of PIP\(_3\), a serine/threonine kinase, Akt (50). Akt has been characterized as a molecule involved in a wide array of cellular processes such as cell survival and proliferation and was recently shown to play a role in actin remodeling and phagocytosis in macrophages and *D. discoideum* (10; 18; 42). Importantly, a putative serine/threonine kinase gene has been identified in *E. histolytica* and was detected in the phagosomes of this parasite using a proteomic analysis (34). Therefore, it is possible that interaction of the chimeric probe with PIP\(_3\) causes sequestration of the endogenous lipids thus preventing them from recruiting downstream protein targets such as Akt and/or other molecules involved in actin assembly and rearrangement. Alternatively, the GFP-
PH{sub}Btk biosensor may directly sequester proteins, which are capable of interaction with this binding domain, thus interrupting relevant signaling cascades. Interestingly, mutations of certain residues within PIP{sub}3-binding probes that do not directly affect their lipid binding were able to abrogate the dominant-negative effects of probe expression (50). These observations, together with the evidence that PH domains can directly interact with other proteins (31; 41), strongly support the idea of protein sequestration by the overexpressed PH domain.

Microscopic observations of GFP-PH{sub}Btk expressing trophozoites that were exposed to hRBCs indicated that the majority of cells adhered to erythrocytes but were unable to complete phagocytosis. This phagocytic defect in GFP-PH{sub}Btk expressing trophozoites resembles wortmannin inhibition of erythrophagocytosis in *E. histolytica* (5; 19; 38). Wortmannin treatment also resulted in a reduction of PIP{sub}3- and PI3P-specific fluorescence at the erythrophagocytic cup as reported here and elsewhere (38). Together, these data indicate that PIP{sub}3 is not required for the adhesion of trophozoites to hRBCs but is critical for the subsequent steps of phagocytosis in *E. histolytica*.

Despite inhibited erythrophagocytosis in GFP-PH{sub}Btk expressing trophozoites, we were able to capture three phagocytic events and characterize PIP{sub}3 kinetics during the uptake of hRBCs. We observed a marked increase in PIP{sub}3–specific fluorescence at the moment of phagosomal closure. Staining of *E. histolytica* trophozoites exposed to hRBCs with a GST-PH{sub}Btk biosensor confirmed the accumulation of PIP{sub}3 at the time of phagosomal closure and showed that
newly sealed phagosomes were devoid of probe-specific fluorescence thus further corroborating the transient nature of PIP₃ partitioning during phagosomal closure. These results are consistent with PIP₃ dynamics during phagocytosis observed in other systems. In *D. discoideum*, a GRP1-PH-GFP probe, that specifically binds to PIP₃, exhibited a peak in fluorescence at the time of phagosome closure around a yeast particle (14). Similarly, PIP₃ was shown to be important for phagosomal closure during erythrophagocytosis in macrophages and uptake of zymosan particles in neutrophils (3; 12). Together, these findings suggest that the role of PIP₃ in the phagocytosis of large particles may be evolutionarily conserved from protozoans to mammalian phagocytes.

An earlier report demonstrated that PI3P accumulated early on the forming erythrophagosomes in *E. histolytica*, which is a unique feature of this parasite, since in mammalian cells PI3P associates with phagosomes only after fusion (38). Here we have shown that PIP₃ also accumulated during early stages of phagosomal biogenesis. These observations raise a question about the importance of the appearance of both of these lipid species in the early erythrophagosomes. It is possible that PIP₃ and PI3P independently recruit different downstream protein targets to the phagosomal membrane and facilitate their subsequent interaction with each other. In support of this, in mammalian cells the Akt serine/threonine kinase is recruited to the plasma membrane by virtue of the interaction of its PH domain with PIP₃ (49). At the membrane, Akt interacts with one of its novel effector proteins, ProF, which contains a FYVE-
finger domain enabling it to bind PI3P at the plasma membrane, where it serves as an adaptor protein for Akt-dependent signaling cascade (16). Thus, simultaneous presence of PIP3 and PI3P at the membrane may allow effective assembly of interacting partners in a signaling pathway. Downstream binding partners of PIP3 in *E. histolytica* are largely unknown. Recent sequencing of the genome of *E. histolytica* and proteomic analysis of phagosomes in this parasites revealed the presence of a number of PH- and FYVE-domain containing guanine nucleotide exchange factors (GEFs) that are known to activate small Rho/Rac GTPases and regulate actin cytoskeleton reorganization (32). Moreover, PH-domain containing RacG was detected in phagocytic cups in *E. histolytica* and was shown to require PIP3 generation for its activation during phagocytosis in *D. discoideum* (32; 47). Therefore, it is conceivable that PIP3 and PI3P segregation at the erythrophagosomal cups allows concomitant recruitment of RhoGEFs and small Rho/Rac GTPases and establishes the specificity of their interaction.

Alternatively, PI3P and PIP3 may facilitate multiple parallel signaling cascades at the phagosomal cups allowing simultaneous activation of cytoskeletal reorganization and vesicle trafficking events. For example, PH-domain containing p21-activated kinase (PAK), known to regulate cytoskeletal reorganization during cell motility (4; 28; 29), was detected in phagosomes isolated from *E. histolytica* by liquid chromatography and mass spectrometry (34). That PI3P may be involved in the process of vesicle trafficking during phagocytosis is illustrated by the presence of Rab5 in prephagosomal vacuoles
(PPV) in *E. histolytica*, since Rab5 effectors are known to possess FYVE-finger domains (43). Thus, it is possible that generation of PIP$_3$ at the erythrophagosomal cups ensures membrane targeting of molecules such as RhoGEFs, Rho/Rac GTPases, and PAKs to initiate cytoskeletal reorganization, while PI3P production recruits molecular machinery involved in vesicular trafficking necessary for phagosome biogenesis.

Visualization of phagocytosing trophozoites using the GST-PH$^{\text{Btk}}$ biosensor indicated that an increase in PIP$_3$-specific fluorescence at the erythrophagosomal cups was accompanied by a drop in fluorescence in the bulk membrane. This localized accumulation of PIP$_3$ at the cups may be attributed to *de novo* production of PIP$_3$ at the site of erythrocyte uptake accompanied by the destruction of PIP$_3$ by SHIP and/or PTEN phosphatases in the rest of the membrane. Conversely, lateral diffusion of PIP$_3$ may cause localized accumulation of this lipid at the erythrophagosomal cups. Although our methodology did not allow us to discriminate between the two possibilities, recent data support the *de novo* synthesis model of PI accumulation. For example, Marshall *et al.* have demonstrated that the time course of PI3-kinase accumulation on phagosomes in macrophages corresponded to that of PIP$_3$, which supports the idea of localized biosynthesis of PIP$_3$ (33). Future studies of PI3-kinase and inositol phosphatase dynamics during endocytosis in *E. histolytica* may clarify how local PIP$_3$ accumulation arises in the phagosomal cups in this parasite.
To gain insight into a mechanism of local restriction of PIP$_3$ at the erythrophagocytic cups, we investigated a possible involvement of detergent-resistant, cholesterol-rich, raft-like membrane microdomains in the erythrophagocytosis in *E. histolytica*. Previously, Laughlin *et al.* demonstrated the existence of lipid rafts in *E. histolytica* and showed that intact rafts are essential for adequate adhesive processes and pinocytosis in this parasite (30). Here we demonstrate that DilC$_{16}$-stained raft-like microdomains also accumulate at the sites of interaction with human erythrocytes in *E. histolytica*. A number of recent studies in mammalian cells implicated lipid rafts as key regulators of phosphoinositide-based signaling that leads to cytoskeletal remodeling. For example, cholesterol depletion abolished PIP$_3$ localization at the phagosomal cups in HL60 neutrophilic cells (12). In growing neurites, it has been shown that intact lipid rafts and PIs are required for the recruitment of the Pyk2/Cbl signaling complex upon stimulation with growth factor (21). Our observation of lipid raft accumulation in the erythrophagocytic cups may support the hypothesis that, like in other systems, lipid rafts function as an intermediate membrane compartment that participates in the regulation of the phagocytosis of hRBCs in *E. histolytica* possibly by providing a structural platform for PI-based signaling. However, the cause-effect relationship between lipid raft accumulation and localized synthesis of PIs during erythrophagocytosis in *E. histolytica* remains to be established.

While both PIP$_3$ and PI3P were shown to participate in the erythrophagocytosis in *E. histolytica* (this study and (38)), neither was found to
play a role in the uptake of 10 kDa fluid-phase marker, FITC-dextran.

Interestingly, it has been previously reported in *E. histolytica* that wortmannin completely inhibited pinocytosis of FITC-dextran (19). It is possible that wortmannin is inhibiting a wide array of PI3-kinase isoforms resulting in the reduction of additional products of PI3-kinase not detectable by our biosensors. In other words, it is possible that inhibition of pinocytosis in the previous studies was due to the depletion of a PI3-kinase product other then PIP₃ or PI3P.

Importantly, our observations of the selective involvement of PIP₃ in different endocytic processes suggest that erythrophagocytosis and pinocytosis of 10 kDa dextran employ different molecular machineries in *E. histolytica*.

In mammalian cells, PIP₃ is very transient and its levels can be reduced by serum starvation (22). Therefore to test the stability of PIP₃ in *E. histolytica* cells, we serum starved trophozoites for 14 hours and then stained with the PIP₃ biosensor. Surprisingly, PIP₃ staining remained relatively unchanged after serum starvation, suggesting that this lipid is present as a highly stable pool in the plasma membrane of *E. histolytica*. Curiously, the concentration of wortmannin required to abolish PIP₃-specific staining exceeded the concentration of wortmannin that eliminated PI3P membrane fluorescence in *E. histolytica* by almost ninefold. While this further demonstrates the stability of PIP₃ in *E. histolytica* cells, it might also suggest that there are multiple wortmannin-responsive targets with unique roles in the maintenance of PI levels and signaling. Curiously, high steady state levels of PIP₃ in *E. histolytica* are
reminiscent of the stability of this lipid seen in a related, non-pathogenic amoeba, *D. discoideum* (59). The reason that PIP₃ exists as a highly persistent pool of lipids in *E. histolytica*, or related single-cell organisms, is not known. In attempt to explain these observations, we describe three important physiological processes in *E. histolytica*, which may require high steady state levels of PIP₃, namely cell motility, nutrient acquisition, and cell survival. It is unlikely that constitutive production/maintenance of PIP₃ in the cell membranes is necessary for adequate and continuous cell movement of *E. histolytica*, since macrophages, which are also highly motile cells, do not exhibit high basal levels of PIP₃ (25, 33). It is possible, however, that PIP₃ may be required for continuous nutrient uptake from the surrounding media by this protozoan. *E. histolytica* trophozoites must remain constitutively phagocytic in order to survive in the host’s large intestine. Even though axenically cultured *E. histolytica* unnaturally rely on pinocytosis for nutrient acquisition (which does not seem to depend on PIP₃), previous studies have demonstrated that phagocytic efficiency remained high in *E. histolytica* during the course of prolonged in vitro subcultivation (20). In mammalian cells, PIP₃ is an important signaling molecule that is critical for cell proliferation and survival and unimpeded production of PIP₃ is believed to be tumorigenic (15). Therefore, the stability of PIP₃ in *E. histolytica* may also be related to the single-cell nature of this parasite, for which rapid and continuous divisions is an important goal. In either case, a better understanding of PI3-kinase function and its associated unique biological features (i.e. early appearance of PI3P on
phagosomes, stability of PIP₃ pool) will provide significant insight into the unique biology of *E. histolytica*.

In summary, this study characterizes for the first time the spatiotemporal aspects of PIP₃ subcellular distribution during endocytosis in *E. histolytica* and demonstrated that this lipid is important for early steps of erythrophagocytosis but is not involved in adhesion or pinocytosis in this parasite. Since these processes represent important virulence functions in *E. histolytica*, this study contributes to our understanding of the molecular events that underlie pathogenic mechanisms in this parasite.

**Future research directions**

Given that PI3-kinase dependent signaling plays a critical role in phagocytosis and, hence, virulence in *E. histolytica*, an in-depth understanding of this process will provide significant insight into the mechanisms of pathogenicity in this parasite. While all known isoforms of mammalian PI3-kinases have been described and classified, the number and classes of PI3-kinase in *E. histolytica* remain undefined. The search of *E. histolytica* genome revealed approximately 15 putative members of PI3-kinase family. The characterization of individual *E. histolytica* PI3-kinases will require the determination of their precise domain structure as well as their substrate specificity. This will allow the experimental characterization of isoform-specific cellular functions of PI3-kinases in *E. histolytica* using targeted ablation of genes encoding specific isoforms. The
development of isoform-specific PI3-kinase inhibitors would also aid in accomplishing this goal. The characterization of the subcellular localization of PI3-kinases and putative inositol phosphatases in *E. histolytica* would complement the findings of PIP$_3$ and PI3P distribution during various endocytic processes and elucidate the dynamics of the interplay between kinases and phosphatases in regulating PIs-dependent signaling.

Currently little is known about the signaling events that occur upstream and downstream of PI3-kinases and their products. The completion of the *E. histolytica* genome provides the opportunity to use genome-wide screens and forward genetics approaches to characterize the upstream and downstream interacting partners of PI3-kinases and phosphoinositides in *E. histolytica*. These findings have the potential to reveal unique aspects of PI3-kinase signaling that might aid in the development of novel effective therapeutic approaches against amoebiases. One unique feature of PI3-kinase signaling that warrants further investigation is the presence of a highly stable pool of PIP$_3$ in *E. histolytica*. The identification of signaling pathways that are constitutively upregulated in response to persistent production/maintenance of membrane PIP$_3$ may uncover novel targets for vaccine and drug development.
References


