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Localization of Phosphatidylinositol (3,4,5)-Trisphosphate to Phagosomes in *Entamoeba histolytica* Achieved Using Glutathione S-Transferase- and Green Fluorescent Protein-Tagged Lipid Biosensors†‡

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*Entamoeba histolytica* is an intestinal protozoan parasite that causes amoebic dysentery and liver abscess. Phagocytosis by the parasite is a critical virulence process, since it is a prerequisite for tissue invasion and establishment of chronic infection. While the roles of many of the proteins that regulate phagocytosis-related signaling events in *E. histolytica* have been characterized, the functions of lipids in this cellular process remain largely unknown in this parasite. In other systems, phosphatidylinositol (3,4,5)-trisphosphate (PIP$_3$), a major product of phosphoinositide 3-kinase (PI3-kinase) activity, is essential for phagocytosis. Pleckstrin homology (PH) domains are protein domains that specifically bind to PIP$_3$. In this study, we utilized glutathione S-transferase (GST)- and green fluorescent protein (GFP)-labeled PH domains as lipid biosensors to characterize the spatiotemporal distribution of PIP$_3$ during various endocytic processes in *E. histolytica*. PIP$_3$-specific biosensors accumulated at extending pseudopodia and in phagosomal cups in trophozoites exposed to erythrocytes but did not localize to pinocytic compartments during the uptake of a fluid-phase marker, dextran. Our results suggest that PIP$_3$ is involved in the early stages of phagosome formation in *E. histolytica*. In addition, we demonstrated that PIP$_3$ exists at high steady-state levels in the plasma membrane of *E. histolytica* and that these levels, unlike those in mammalian cells, are not abolished by serum withdrawal. Finally, expression of a PH domain in trophozoites inhibited erythrophagocytosis and enhanced motility, providing genetic evidence supporting the role of PI3-kinase signaling in these processes in *E. histolytica*.

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*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 and is associated with low basic hygiene standards and a lack of water sanitation (reviewed in reference 20). Since *E. histolytica* is a human parasite (40), improvements in sanitation may help prevent the fecal-oral spread of this pathogen; however, overpopulation, scarcity of clean water, and socioeconomic shortcomings impede the progress of such improvements in developing countries (36). This lack of progress supports an elevated need for the development of improved prevention, diagnosis, and treatment for dysentery caused by *E. histolytica*. This requires a better understanding of the basic biology of this parasite.

*E. histolytica* infections are contracted by ingestion of its multinucleate infective cysts from fecally contaminated food or water (reviewed in reference 39). Upon exstavation in the small intestine, motile trophozoites move to the bowel lumen, where bacteria, erythrocytes, and host cell debris serve as food sources which are taken up by phagocytosis. Infection is established when trophozoites adhere to the intestinal wall, destroy colonic epithelium, and occasionally disseminate via the hemogenous route to extraintestinal sites.

Phagocytosis is recognized as an important virulence function in this parasite. For example, several studies suggest a connection between exposure to intestinal bacteria and increased virulence in *E. histolytica* (3, 46). Furthermore, transcriptional profiling of *E. histolytica* exposed to *Escherichia coli* revealed increased gene expression of a protein kinase, an ABC transporter, a Rho family GTPase, and Hsp90, which may collectively modulate virulence in this parasite (6). Finally, phagocytosis-deficient mutants of *E. histolytica* exhibit reduced virulence (27), and an avirulent *Entamoeba* species, *E. dispar*, carries out limited phagocytosis compared to the virulent species (28). Therefore, understanding the molecular mechanisms of phagocytosis in *E. histolytica* may provide insight into factors that contribute to virulence.

Two recent proteomic screens of purified *E. histolytica* phagosomes have revealed proteins that may be involved in the processes of phagosome biogenesis in this parasite (22, 26). A putative phosphoinositide 3 kinase (PI3-kinase) was identified as one of the signaling proteins that physically associate with *E. histolytica* phagosomes. PI3-kinases belong to a family of proteins that generate signaling phosphoinositides (PIs) phosphorylated on hydroxyl groups. These include phosphatidylinositol 3-phosphate (PI3P), phosphatidylinositol (3,4)-bisphosphate (PIP$_2$), and phosphatidylinositol (3,4,5)-trisphosphate (PIP$_3$) (10). Phosphorylated PIs are capable of regulating phagocytosis by recruiting proteins via specific lipid-recognition domains. Examples of such protein motifs include FYVE finger do-
mains, which specifically bind PI3P and pleckstrin homology (PH) domains, some of which specifically interact with PIP3 (reviewed in reference 17). The possibility that PI3-kinase and its products may be involved in the regulation of phagocytosis in *E. histolytica* is also supported by the finding that small-molecule inhibitors of PI3-kinase block uptake of phagocytic targets (2, 14, 30).

In a previous study, a recombinant glutathione S-transferase (GST)-labeled FYVE finger protein domain was used to localize PI3P in *E. histolytica* trophozoites (30). It was demonstrated that PI3P accumulated in forming erythropagosomal cups of *E. histolytica*. This localization was confirmed by Nakada-Tsukui et al. (24) using live-cell imaging of trophozoites expressing green fluorescent protein (GFP)-labeled FYVE-finger domains. GFP-labeled PH domains have demonstrated that PIP3 regulates phagocytosis in neutrophils (7), macrophages (1), and a nonpathogenic soil amoebo, *Dictyostelium discoideum* (9, 13). However, little is known about the evolution of PIP3 on cellular membranes in *E. histolytica*, particularly during phagocytosis. Therefore, in this study, we investigated the spatiotemporal characteristics of PIP3 distribution during endocytosis using both GFP- and GST-tagged biosensors containing a PH domain derived from Bruton's tyrosine kinase (Btk). This PH domain specifically binds to PIP3 (32, 35).

**MATERIALS AND METHODS**

**Strains and culture conditions.** Entamoeba histolytica trophozoites (strain HM-1:IMSS) were cultured axenically in TYI-S-33 (8) in glass screw-cap tubes at 37°C.

**Generation of GST-PI3H.** The biosensor construct was obtained from a pEGFP-N1 parent plasmid modified by the manufacturer (SignaGen Laboratories, Gaithersburg, MD) to contain the cDNA encoding the Bruton's tyrosine kinase (Btk) pleckstrin homology (PH) domain cloned between the EcoRI and BamH1 sites upstream of the sequences encoding enhanced green fluorescent protein (EGFP, hereinafter referred to as GFP). The DNA encoding PH-EGFP was PCR amplified from the plasmid template using the following pair of primers: 5′-CC GATCTCTGAAAGAGGGGCG-3′ and 5′-CCGGAATCTGGTTTGAACG-3′. The PCR product was subcloned into the pCR2.1-TOPO plasmid vector (Invitrogen, Carlsbad, CA). After digestion with BamH1 and EcoRI, the PH domain-encoding DNA fragment was ligated into the polylinker region downstream of and in frame with the sequence encoding GST in the pGEX-5x-1 domain-encoding DNA fragment was ligated into the polylinker region downstream of and in frame with the sequence encoding GST in the pGEX-5x-1 expression vector (Amersham Biosciences, Piscataway, NJ). Successful construct generation was confirmed by restriction enzyme analysis and sequencing. GST and GST-PI3H fusion proteins were expressed in Escherichia coli BL21 (Amersham GE Biosciences, Piscataway, NJ) and purified using glutathione-Sepharose affinity chromatography as previously described (37). The purity of GST and GST fusion proteins was assessed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and silver staining as described previously (29). Western blot analysis was performed as described previously (44) to verify the authenticity of the GFP-tagged protein using a 1:10,000 dilution of mouse anti-GFP antibodies (Invitrogen). Time-lapse video microscopy. For time-lapse video confocal microscopy of live trophozoites expressing GFP-PI3H or GFP, cells were incubated on ice for 10 min to remove them from the glass surface of the culture tube, pelleted by centrifugation at 500 × g for 5 min at 25°C, and placed in warmed phosphate-buffered saline (PBS) in glass chamber slides for observation using a LSM 510 laser confocal microscope (Carl Zeiss Inc., Thornwood, NY). To observe phagocytosis, GFP-PI3H-expressing cells and control trophozoites were exposed to human red blood cells (hRBCs) (U.S. Biologicals, Swampscott, MA) during the imaging session. Time-lapse images were collected at 5-s intervals. The instant of adhesion to an erythrocyte was designated time zero (t0). Image analysis was carried out via nyz plane images generated with Image J software (National Institute of Health, Bethesda, MD). A supplemental movie was compiled using QuickTime Pro (Apple Computer Inc., Cupertino, CA).

**Microscopy.** For immunofluorescence (IF) microscopy using GST-PI3H, *E. histolytica* trophozoites were incubated with hRBCs (ratio of *E. histolytica* to hRBCs = 1:100) for 1 min or with lysine-fixable tetramethyl rhodamine isothio- cyanate (TRITC)-dextran (0.3 mg ml−1) (10 kDa; Invitrogen) for 2, 10, 30, or 60 min. Cells were subsequently fixed, permeabilized, and stained with recombinant GST or GST-PI3H as described previously (30). Trophozoites stained with GST alone served as controls. Alexa Fluor 488-conjugated goat anti-GST antibody (1:1,000) (Rockland Immunochemicals, Gilbertsville, PA) was used for GST-PI3H immunodetection.

For comparison, wild-type cells were also stained with anti-PiP3, antibody (Echelon Biosciences, Salt Lake City, UT) per the manufacturer's protocol, with modifications. Briefly, cells were fixed in glass screw-cap tubes for 24 h in 4% (vol/vol) paraformaldehyde in TYI-S-33 at 37°C. Cells were scraped from the glass and harvested by centrifugation (500 × g, 5 min, 25°C). Cells were washed three times in Tris-buffered saline (TBS) and then permeabilized in 0.5% (vol/vol) saponin in phosphate-buffered saline (PBS) for 15 min at room temperature. Cells were washed three times in TBS and blocked for 30 min at 37°C in 1% (w/v) bovine serum albumin (BSA) and 0.1% (v/v) saponin in PBS. The supernatant was removed and 50 μl trypan blue (0.5 mg ml−1) was added to the cells and washed three times in 1% (vol/vol) goat serum/TBS. Cells were then incubated with Alexa Fluor 488 goat anti-mouse/TBS (1:1,000) for 30 min at 37°C.

To confirm the authenticity of GST-PI3H staining, cells were treated with a range of concentrations (60, 100, 250, and 500 nM) of the PI-3 kinase inhibitor wortmannin (Sigma Chemicals, St. Louis, MO) for 10 min prior to fixation and decoration with the PI-P, biosensor. Cell viability after wortmannin treatment was estimated using trypan blue exclusion (0.5 mg ml−1).

Cells stained with GST-PI3H or anti-PiP3, antibody were mounted in glycerol/ PBS (1:1) solution on microscope slides and observed using a Zeiss LSM 510 confocal microscope.

**Measurement of erythrophagocytosis and fluid-phase pinocytosis.** Measurement of phagocytosis of hRBCs in *E. histolytica* cell lines were carried out according to the methods of Voigt et al. (43). Fluid-phase pinocytosis was measured using the fluorescent fluid-phase marker FITC (fluorescein isothiocyanate)-dextran (10 kDa; 2 mg ml−1) (Sigma Chemicals) as previously described (45). A correction for background GFP fluorescence was achieved by subtracting the initial (time zero) fluorescence values from all other fluorescence values.

**Measurement of adhesion to erythrocytes.** Adhesion of *E. histolytica* amoebae to erythrocytes was assayed using a previously described rosette formation assay (33). Briefly, trophozoites (1 × 106) and erythrocytes (1 × 109) were mixed in 500 μl of TYI-S-33, centrifuged at 9000 × g, 4°C, 5 min, and resuspended in 30 min on ice. Following incubation, 450 μl of the supernatant was removed and 50 μl trypan blue was added. A portion of the resulting mixture (10 μl) was used for counting on a hemacytometer, and at least 200 trophozoites were scored per trial. Adherent amoebae were defined as those with three or more bound erythrocytes (33).
Motility assay. Motility assays were carried out according to the methods of Zaki et al. (47), with modifications. To prepare the motility chambers, 8 ml of complete TYI-S-33 medium, supplemented with 0.75% (wt/vol) agarose, was poured into 60-mm petri dishes and allowed to solidify at room temperature. Transgenic trophozoites were induced to express GFP or GFP-PHBtk by tetracycline treatment for 24 h. Wild-type and transgenic trophozoites were then incubated in incomplete medium lacking tryptone and yeast extract for 1 h. Following this incubation, 5 x 10^5 cells (suspended in incomplete medium) were placed in a trough (2 by 30 mm) that had been cut into the solidified complete medium. A coverslip (22 by 40 mm) was placed over the trough, and the plate was incubated at 37°C for 1 h in 5% CO₂. Migrations were visualized using a Zeiss LSM 510 confocal microscope. Measurements of migration distance were determined using Zeiss LSM 510 image analysis software.

Statistical analyses. All values are given as means ± 1 standard deviation (SD). Statistical analyses were performed using GraphPad Instat V.3 with one-way analysis of variance (ANOVA) and a Tukey-Kramer multiple comparison test. *P* values less than 0.001 were considered extremely significant. *P* values less than 0.01 were considered highly significant, and *P* values between 0.01 and 0.05 were considered statistically significant.

RESULTS

A GFP-tagged PH domain can be used to localize PIP₃ in *E. histolytica* trophozoites. In order to observe the spatial and temporal localization of PIP₃ in *E. histolytica*, we generated cell lines that stably expressed GFP or GFP-PHBtk. Expression of the exogenous proteins was confirmed by Western blotting of cell lysates prepared from the transfected trophozoites with anti-GFP antibody (Fig. 1). A number of protein bands nonspecifically reacting with the anti-GFP antibody were also visible on the Western blots (Fig. 1). However, it is unlikely that these proteins were degradation products of the exogenous protein, since they were also evident before induction with tetracycline.

The dynamic distribution of PIP₃ was then observed by microscopy in the transformed cell lines. First, microscopy revealed that expression of GFP or GFP-PHBtk was variable among transformed trophozoites within the population (Fig. 2; A to C).

**FIG. 1.** Western blot analysis using anti-GFP antibody of lysates prepared from cells expressing GFP or GFP-PHBtk. Lysates were prepared from cells before (−) and after (+) induction with tetracycline (tet). GFP and GFP-PHBtk exogenous proteins are indicated with arrows (right) and asterisks. Molecular masses are shown on the left.

**FIG. 2.** GFP-PHBtk is localized to extending pseudopodia in randomly moving *E. histolytica* trophozoites. (A to C) Live-cell fluorescence microscopy of wild-type cells (A) and trophozoites induced to express GFP (B) or GFP-PHBtk (C). (D to F) Corresponding DIC images. GFP-expressing control trophozoites exhibited cytoplasmic fluorescence, which did not localize to extending pseudopodia (arrows in panels B and E). In trophozoites expressing GFP-PHBtk, PIP₃-specific fluorescence was observed in pseudopodia during random cell movement (arrows in panels C and F). Bars, 10 μm (A and B) or 20 μm (C).
also, see Movie S1 in the supplemental material, which shows GFP-PH\textsuperscript{Btk}\,-expressing cells). Others have observed variable expression of GFP-labeled proteins in populations of transformed trophozoites. For example, trophozoites expressing GFP-tagged FYVE finger domains, derived from an \textit{E. histolytica} protein, EhFP4, exhibited variable expression, as evidenced by differences in fluorescence intensity from cell to cell (24). Second, observation of randomly moving trophozoites revealed a diffuse intracellular staining in quiescent cells and a prominent localization of the PIP\textsubscript{3}-specific probe in extending pseudopodia (Fig. 2C and F; also, see Movie S1 in the supplemental material). In contrast, control cells expressing GFP alone exhibited cytosolic fluorescence, which was excluded from forming pseudopodia (Fig. 2B and E, arrows). Phagocytosis and cell motility share many features, including the requirements for membrane extension formation and localized

FIG. 3. Time-lapse confocal images of GFP-PH\textsuperscript{Btk}\,-expressing \textit{E. histolytica} trophozoites during phagocytosis of hRBCs. Transformed \textit{E. histolytica} trophozoites were exposed to hRBCs, and images were collected in a single confocal plane at the indicated time points (seconds). Adhesion (arrow) of \textit{E. histolytica} to erythrocytes was defined as time zero \((t_0)\), and subsequent images represent the uptake of a single particle: extension and closure of pseudopodia around the erythrocyte \((t_{30} \text{ to } t_{50})\) and internalization of the newly sealed phagosome \((t_{60} \text{ to } t_{70})\). The time-lapse series is indicative of at least three independent phagocytic events. Both DIC and fluorescence images are shown. Bars, 10 \(\mu m\).
actin polymerization (23). Therefore, localization of PIP3 to randomly extending pseudopodia in *E. histolytica* was not surprising. Untransfected wild-type amoebae exhibited minimal background fluorescence (Fig. 2A).

To gain insight into the timing of PIP3 evolution during phagocytosis of hRBCs, *E. histolytica* trophozoites expressing GFP-PHBtk were exposed to erythrocytes and visualized by time-lapse fluorescence imaging. The ingestion of a single erythrocyte occurred over a 60- to 80-s 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 and phagosomal closure with subsequent internalization of the newly sealed phagosome (Fig. 3).

Accumulation of GFP-PHBtk was not evident at the site of erythrocyte attachment, suggesting that PIP3 is not involved in the earliest stages of particle binding and adhesion (Fig. 3, t0). However, a peak in PIP3 fluorescence intensity was detected during the formation of deep and nearly sealed erythrocyte-containing membrane invaginations (Fig. 3, t40). The rise in PIP3-specific signal at the cup was most obvious in three-dimensional plots generated from corresponding single-plane confocal images, in which fluorescence intensity is represented as the height of the *xyz* plot (Fig. 4, t40). This accumulation was transient, lasting less than 10 s, and was followed by a rapid dissipation of the signal during the internalization of the phagosome (Fig. 3 and 4, t50 to t70). These data are consistent with known PIP3 dynamics that occur during the phagocytosis of particles in neutrophils (7) and *D. discoideum* (9).

Expression of GFP-PHBtk decreases erythrophagocytosis but has no effect on adhesion to targets or pinocytosis. Microscopic comparison of erythrophagocytosis in GFP-PHBtk-expressing trophozoites with that in wild-type or GFP control cells indicated that actual phagocytic events were rare in the transgenic cells. This suggested a functional defect in phagocytosis and prompted us to quantify phagocytosis in *E. histolytica* cells expressing GFP-PHBtk.

Trophozoites were exposed to hRBCs for 10 min, after which extracellular erythrocytes were lysed hypotonically with distilled water and the level of ingested heme was measured by spectrophotometry. Erythrophagocytosis in GFP-PHBtk-expressing trophozoites was inhibited by 78% and 69% compared to the wild-type and GFP control cells, respectively (Fig. 5A). This suggests that expression of the GFP-tagged PH domain exerts a dominant negative effect on this cellular process.

To gain further insight into the phagocytosis defect, we measured the ability of wild-type and transgenic cell lines to adhere to erythrocytes using a standard rosette assay (33). Adhesion is considered an initial step in phagocytosis. Interestingly, cells expressing GFP-PHBtk bound erythrocytes as efficiently as wild-type or GFP-expressing control cells (Fig. 5B). Therefore, it is likely that only later steps in phagocytosis, such as cup formation and closure, are inhibited by GFP-PHBtk expression. These data are consistent with microscopy data indicating that the GFP signal was not enhanced at the site of adhesion to erythrocytes (Fig. 3 and 4, t0). These data are also consistent with a previous observation that chemical inhibitors of PI3-
kinase can block internalization but not binding of erythrocytes
(30). To determine if the cell line expressing GFP-PH Btk possessed a general endocytic defect, we measured uptake of a 10-kDa fluid-phase marker, FITC-dextran. Interestingly, uptake of this marker was not affected by the expression of the GFP-PHBtk (Fig. 5C). Importantly, the differential effect of GFP-PHBtk expression on phagocytosis versus pinocytosis suggested that inhibition of phagocytosis was specific.

Expression of GFP-PHBtk enhances motility in E. histolytica trophozoites. Since the GFP-tagged biosensor accumulated in the membrane extensions of randomly moving trophozoites (Fig. 2; also, see Movie S1 in the supplemental material) and since PIs are involved in regulating cell motility in other systems (reviewed in reference 18), we also examined motility in E. histolytica trophozoites expressing GFP-PHBtk. Interestingly, under-agar motility assays indicated that cells expressing the PIP3 biosensor displayed enhanced motility compared to GFP-expressing or wild-type cells (Fig. 6), supporting the notion that PIP3-based signaling is important in motility in E. histolytica and that such signaling pathways may be altered by the expression of GFP-PHBtk.

A GST-tagged PH domain can be used as a PIP3 biosensor in E. histolytica trophozoites. Since expression of GFP-PH Btk altered cellular functions in E. histolytica trophozoites, it was necessary to confirm the localization of PIP3 using untransformed cells. It was recently shown that recombinant glutathione-S-transferase (GST)-labeled FYVE finger protein domains and fluorescent anti-GST antibody could be used to localize PI3P in E. histolytica trophozoites (30). This provided the impetus to develop an analogous method to localize intracellular PIP3, using a GST-tagged PHBtk domain (GST-PHBtk). Others have shown that recombinant GST-PHBtk can bind specifically to PIP3 in vitro (32, 35). Importantly, Salim et al. (35) also demonstrated that fluorescent anti-GST antibody does not interfere with the interaction of GST-PHBtk and liposomes containing PIP3. Together, these data suggest that the GST-tagged pleckstrin homology domain of Btk is conformationally and functionally intact and supports its use as a lipid biosensor.

Bacterial expression of GST and GST-PHBtk and subsequent affinity purification yielded 28-kDa and 49-kDa proteins, respectively (Fig. 7A). The larger protein (49 kDa) had a molecular weight equivalent to the combined molecular weights of the GST tag and the PHBtk domain. The authenticity of the GST and GST-PHBtk recombinant proteins was ver-

FIG. 5. Expression of GFP-PHBtk inhibited erythrophagocytosis in E. histolytica but did not affect adhesion to hRBCs or fluid-phase pinocytosis. In all cases, data are presented as percentage of the wild-type values, which were arbitrarily set to 100%. (A) Quantification of erythrophagocytosis in wild-type cells and cells expressing GFP or GFP-PHBtk. A significant decrease in erythrophagocytosis (***, P < 0.001) was observed for GFP-PHBtk-expressing trophozoites compared to wild-type or GFP-expressing cells. (B) Quantification of adhesion in wild-type cells and cells expressing GFP or GFP-PHBtk. Adhesion to hRBCs was not impeded by GFP-PHBtk expression. (C) Quantification of fluid-phase pinocytosis in wild-type cells and cells expressing GFP or GFP-PHBtk. Uptake of fluid phase was not inhibited by GFP-PHBtk expression. The data are means ± SDs from three independent experiments.

FIG. 6. Motility is enhanced in E. histolytica trophozoites expressing GFP-PHBtk. Expression of GFP-PHBtk enhanced the motility of trophozoites in a statistically significant fashion compared to GFP-expressing control and parental wild-type cells (*, P < 0.5; **, P < 0.01). The data are means ± SDs from four independent experiments.

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ified by Western blot analysis using anti-GST antibodies (Fig. 7B).

To test the utility of the PIP₃ biosensor, *E. histolytica* trophozoites were fixed, permeabilized, and stained with GST-PH₃tk or GST as previously described (30). Imaging revealed that the biosensor uniformly decorated plasma membranes, producing a high-intensity signal along the cell periphery and giving it a ring-like appearance (Fig. 8A, panel ii). Some intracellular staining was also evident (Fig. 8A, panel ii). This ring-like fluorescence pattern was different from that observed in trophozoites expressing the GFP-tagged probe. However, images of GST-PH₃tk-stained cells were obtained by confocal optical sectioning and represent a single confocal plane, while images of transgenic trophozoites were obtained in live-cell mode and represent total fluorescence. Therefore, the dissimilarity is likely the result of various image capture methods. A similar difference was noted (24) when trophozoites stained with GST-tagged FYVE finger domains (30) were compared to those expressing GFP-tagged FYVE finger domains (24). Cells stained with the control GST protein lacking a PH₃tk domain exhibited minimal fluorescence (Fig. 8A, panel i).

We also compared the pattern of staining obtained with GST-PH₃tk to that obtained by standard immunofluorescence microscopy using an anti-PIP₃ antibody. Decoration of trophozoites with an anti-PIP₃ antibody resulted in uniform peripheral fluorescence with some intracellular staining (Fig. 8B, panel ii). This pattern was similar to that of GST-PH₃tk-stained cells, supporting the authenticity of the staining pattern with the recombinant probe.

As an additional test for the specificity of biosensor-PIP₃ interaction, we utilized a known inhibitor of PI3-kinase activity, wortmannin. Interaction of a PI3P-specific probe, GST-2XFYVE, with trophozoite membranes was maximally inhibited by 60 nM wortmannin (30). Therefore, this concentration was used as a starting point to test the response of the PIP₃-specific probe, GST-PH₃tk, to PI3-kinase inhibition. *E. histolytica* trophozoites were treated with increasing concentrations of wortmannin (60 nM, 100 nM, 250 nM, and 500 nM) for 15 or 30 min prior to fixation and decoration with the biosensor, and the intensity of fluorescence was measured. Treatment with 60 nM wortmannin did not significantly inhibit staining with GST-PH₃tk (data not shown). Staining with the GST-tagged probe was not inhibited by treatment with 100 nM wortmannin for 15 min and was slightly inhibited by treatment with 100 nM wortmannin for 30 min (Fig. 9A, panels i and iv, and B). Treatment with higher concentrations of wortmannin inhibited GST-PH₃tk staining throughout trophozoites in a dose- and time-dependent manner (Fig. 9A, panels ii, iii, v, and vi, and B), suggesting that GST-PH₃tk authentically binds to products of PI3-kinase. Importantly, the viability of trophozoites exposed to wortmannin was not affected, since trypan blue exclusion demonstrated that 96.7% cells remained viable.

![FIG. 7. Purification of GST and the GST-PH₃tk fusion protein. (A) SDS-PAGE and silver stain of GST and GST-PH₃tk protein preparations after affinity purification. (B) Western blot analysis using anti-GST antibody of purified GST and GST-PH₃tk, verifying the authenticity of the GST-tagged chimera. Molecular masses (M) are shown to the left of each panel.](image-url)

![FIG. 8. Localization of PIP₃ in untransfected cells. (A) *E. histolytica* trophozoites were stained with GST-PH₃tk and fluorescent anti-GST antibody. GST-PH₃tk uniformly decorated plasma membranes, giving rise to a ringed appearance of the cell (ii). Some intracellular staining is also evident (ii). As controls, cells were also probed with GST, which did not exhibit any staining (i). Corresponding DIC images are shown (iii and iv). (B) Immunofluorescence staining of *E. histolytica* trophozoites with an anti-PIP₃ antibody (i and ii) or secondary antibody alone (control; iii). Both a field of cells (i) and an individual cell (ii) are shown. Corresponding DIC images are shown (iv to vi). Bars, 10 μm.](image-url)
after treatment with 500 nM wortmannin for 30 min (data not shown). In other systems, similarly high concentrations of wortmannin (200 nM [16] and 500 nM [Btk-PH redistribution assay; Thermo Scientific]) were used to abolish the interaction between PIP3 and PH domains.

PIP3 is virtually undetectable in quiescent mammalian cells and transiently increases upon stimulation with growth factors and chemokines (38). Since trophozoites grown in culture medium, which is supplemented with adult bovine serum, may be exposed to such growth factors, we tested whether serum withdrawal would alter the localization or intensity of biosensor staining.

E. histolytica cells were cultured in serum-free medium for 14 h, fixed, stained with GST-PHBtk, and visualized using fluorescence microscopy. Serum starvation inhibited GST-PH Btk staining in the plasma membrane only slightly (Fig. 10A). Quantitative measurement of membrane fluorescence indicated no statistical differences between populations of cells that were grown with and without serum (Fig. 10B). Similarly, probe-specific staining was not altered by a 1 h of incubation of trophozoites in PBS (data not shown). Importantly, trophozoites remained viable during 14 h of serum starvation or 1 h of PBS incubation (data not shown). While it is possible that these conditions were not sufficient to induce quiescence in trophozoites, the data suggest that E. histolytica exhibits high steady-state levels of PIP3.

The recombinant PIP3 biosensor accumulates in early phagosomal structures but not in fluid-filled pinosomes in E. histolytica. To confirm the subcellular localization of PIP3 during phagocytosis obtained using expression of GFP-PH-Btk, E. histolytica trophozoites were exposed to hRBCs for 1 min. Subsequently, the cells were fixed, permeabilized, and stained with GST-PH-Btk or with the GST control protein as previously described (30). Different stages of erythrocyte uptake were captured using laser-scanning confocal microscopy. GST-PH-Btk labeled membranes were associated with invaginations surrounding hRBCs (Fig. 11A) and nearly sealed erythropagocytic cups (Fig. 11B). However, no
probe-specific staining was observed in internalized erythrophagosomes located just beneath the plasma membrane (Fig. 11C and H, arrowheads), which suggests that PIP₃ dissipates from the phagosomal membrane after closure is complete. These data are consistent with those obtained by live-cell microscopy of cells expressing GFP-PHBtk (Fig. 3 and 4).

Erythrocyte membranes were not stained with the GST-PHBtk, as evidenced by the extracellular hRBCs captured in the same confocal plane as the labeled *E. histolytica* cell (Fig. 11G, asterisk), demonstrating that GST-PHBtk staining was specific for *E. histolytica*. Control cells that were stained with the GST protein alone did not exhibit any fluorescence at the sites of interaction with erythrocytes (Fig. 11D and I, arrowheads). As expected, erythrophagosomal cups were rare in wortmannin-treated trophozoites; however, partially engulfed hRBCs were occasionally observed (Fig. 11E and J, arrowhead). In these instances, GST-PHBtk staining was not visible at the cup (Fig. 11E, arrowhead). Together, these results suggest that PIP₃ localizes to phagosomes during formation of the cup up to the point of closure. The data also suggest that PIP₃ may not participate in later stages (maturation) of phagocytosis in *E. histolytica*.

To confirm a lack of involvement of PIP₃ in fluid-phase uptake, *E. histolytica* trophozoites were exposed to lysine-fixable tetramethyl rhodamine isothiocyanate (TRITC)-dextran (10 kDa) for 2, 5, 10, and 60 min prior to fixation and staining with GST-PHBtk. Similar to a previous report describing a lack of association of PI3P with pinosomes (30), the PIP₃-specific probe (Fig. 12B) did not colocalize with fluid-phase compartments (Fig. 12A) at any time (only the 60-min time point is shown). This observation is in accordance with data showing
that expression of GFP-PHBtk did not inhibit uptake of FITC-dextran (Fig. 5C). Together, these data indicate that PIP3 may not be involved in the uptake of fluid phase from the surrounding medium.

**DISCUSSION**

In this study we utilized GFP- and GST-labeled PH domains to examine the subcellular localization of PIP3 in *E. histolytica*. We showed that these PIP3-specific biosensors accumulated in membrane extensions in *E. histolytica* during random movement and phagocytosis. Microscopy also revealed that trophozoites may possess high steady-state levels of PIP3. We also demonstrated that expression of the GFP-tagged PHBtk domain inhibited phagocytosis and enhanced motility in this pathogen, supporting the role of PIP3 in these cellular functions. Since the GST-tagged probe did not colocalize with fluid-filled vesicles and since expression of the GFP-tagged probe did not inhibit trophozoite-erythrocyte interactions or uptake of fluid-phase, PIP3 may not be involved in adhesion of trophozoites to hRBCs or in fluid-phase pinocytosis in *E. histolytica*.

It was previously demonstrated that P13P was present on early erythrophagosomes in *E. histolytica* (30). In the present study we showed that PIP3 similarly accumulates during phagosome biogenesis. These observations raise the question of why both of these lipids appear early in phagocytosis in some biogenesis. These observations raise the question of why study we showed that PIP3 similarly accumulates during phagocytosis in *E. histolytica* early erythrophagosomes in hRBCs or in fluid-phase pinocytosis in *E. histolytica*. Membrane staining is undetectable in the control cells stained with GST protein lacking the PH domain (arrowhead). (E) Pretreatment with wortmannin (500 nM; 30 min) abolished GST-PHBtk staining of erythrophagosomes and arrested the extension of the pseudopodia around the erythrocyte (arrowhead). (F to J) Corresponding DIC images. The asterisk in panel G shows an unstained hRBC. Bars, 10 μm.

 FIG. 11. GST-PHBtk decorates erythrophagosomal cups in *E. histolytica*. Trophozoites were challenged with hRBCs for 1 min and stained with GST-PHBtk. The GST tag was visualized using Texas Red-conjugated goat anti-GST antibody. (A) PIP3-specific fluorescence is enhanced in the membrane extending around the erythrocyte (arrow). (B) PIP3-specific fluorescence is enhanced in nearly sealed phagosomes (arrow). However, PIP3-specific fluorescence is not observed around uninternalized hRBCs. (C) PIP3-specific membrane staining is not observed around completely internalized hRBCs (arrowheads). (D) Membrane staining is undetectable in the control cells stained with GST protein lacking the PH domain (arrowhead). (E) Pretreatment with wortmannin (500 nM; 30 min) abolished GST-PHBtk staining of erythrophagosomes and arrested the extension of the pseudopodia around the erythrocyte (arrowhead). (F to J) Corresponding DIC images. The asterisk in panel G shows an unstained hRBC. Bars, 10 μm.

Although variable expression of GFP-PHBtk was observed among transgenic trophozoites within a single population, measurements of the whole population of mutants revealed a phagocytosis defect. These data suggest that it might be possible to perturb PI-based signaling by overexpression of protein domains that bind to phosphorylated products of PIP3. The observation also indicates that phagocytosis in *E. histolytica* is very sensitive to such perturbation. Importantly, uptake of fluid phase pinocytosis was not inhibited in the same population of transformants, which supports the authenticity of the observed phagocytic defect. A similar reduction in the uptake of phagocytic targets, but not of a fluid-phase marker, was observed after expression of GFP-tagged FYVE finger domains in *E. histolytica* (24). The latter study further supports the notion that overexpression of lipid binding domains can be used to study PI-based signaling in *E. histolytica*.

In other systems, overexpression of PH-containing proteins does indeed interfere with PI-based signaling (5, 42). For example, Varnai et al. demonstrated that expression of GFP-PHBtk in fibroblasts inhibited activation of Akt, a well-characterized downstream target of PIP3 (42). It is possible that interaction of the chimeric probe with PIP3 causes titration of endogenous lipids, thus preventing the recruitment of downstream protein targets such as Akt. In addition to lipids, PH domains have been shown to interact with a variety of proteins in *vitro*; whether they interact with such proteins *in vivo* has only been confirmed for a few candidates (21, 34). Therefore, in addition to titration of lipid, expressed PHBtk may also sequester proteins that normally interact with PH domains. This, too, would interrupt relevant signaling cascades.

The activity of P13-kinase is balanced by the activity of phosphatases, such as PTEN or SHIP, which can remove the phosphate moiety from PIP3, converting it to PIP2 (reviewed in
Therefore, titration of PIP3 by GFP-PHBtk may not only inhibit recruitment of Akt but also block the action of phosphatases, resulting in aberrantly high cellular levels of PIP3 and low cellular levels of PIP2. Turnover of PIP3 is critical to phagocytosis, since PIP3 is proposed to regulate pseudopod extension, whereas PIP2 is proposed to regulate actin remodeling (reviewed in reference 25). Thus, it is conceivable that altering PIP3/PIP2 ratios could inhibit phagocytosis, as seen in the GFP-PHBtk-expressing cell line.

How is it possible, however, that expression of a PH domain inhibits phagocytosis while enhancing another PI-dependent cellular function, namely, motility? Interestingly, loss of PTEN function is common during tumorigenesis (4), and this deficit promotes motility and invasiveness. In such tumor cells, PIP3 is not converted to PIP2, which leads to persistent PIP3-specific signaling and enhanced motility. Similarly, if the chimera protected PIP3 from the action of cellular phosphatases in trophozoites, cell movement might have been promoted.

In mammalian cells, PIP3 is transient and its levels can be reduced by serum starvation (38). Surprisingly, PIP3 staining was only slightly inhibited after serum starvation in E. histolytica trophozoites. Although we cannot rule out the possibility that this culture condition did not induce a quiescence-like state in E. histolytica, the data suggest that PIP3 exhibits high steady-state levels in this organism. Since turnover of PIP3 is important for cellular processes such as motility (4), apparent high steady-state levels of PIP3 in E. histolytica might be achieved by a continuous rapid phosphorylation and dephosphorylation cycle.

Although different from levels in mammalian cells, high steady-state levels of PIP3 in E. histolytica are similar to those seen in a related, nonpathogenic amoeba, D. discoideum (48). It is not known if PIP3 in D. discoideum is concentrated at the plasma membrane, since all localization studies were conducted with GFP-PH domain-expressing amoebae and live-cell imaging (9, 48). The reason that PIP3 exists as a highly persistent pool of lipids in these organisms is not known. However, several important physiological processes in these lower eukaryotes may require such high steady-state levels of PIP3. Given its role in phagocytosis, PIP3 may be required for the continuous uptake of nutrients, a common characteristic of these protozoa. PI3-kinase signaling also plays a role in cell proliferation and survival (reviewed in reference 11). Therefore, the stability of PIP3 in E. histolytica and D. discoideum may also be related to their single-cell nature, for which rapid and continuous divisions are an important goal.

In summary, this study characterized for the first time the spatiotemporal distribution of PIP3 in E. histolytica and demonstrated that this lipid is important for early steps of erythrophagocytosis. Since this process represents an important vir-

FIG. 12. GST-PH\textsuperscript{Btk} staining does not colocalize with fluid-filled endosomes. (A and B) E. histolytica trophozoites were exposed to a fluid-phase marker, TRITC-dextran (A, red) for 60 min and then stained with GST-PH\textsuperscript{Btk}, followed by Alexa Fluor 488-conjugated goat anti-GST secondary antibody (B, green). (C) The merged image shows that the PIP3 biosensor does not colocalize with fluid-filled endosomes. (D) Corresponding DIC image. Bars, 10 μm.
ulence function in *E. histolytica*, this study contributes to our understanding of the molecular events that underlie pathogenic mechanisms in this parasite. The downstream interacting partners of phosphoinositides in *E. histolytica* are not known. However, identification and functional analyses of PI3P- and PIP_2_-binding proteins will provide additional insight into pathogenesis. Finally, given that PI3-kinase based signaling may be perturbed in the cell line expressing GFP-PI(1)3, further study of this mutant will provide the opportunity to use a genetic approach to gain insight into cellular signaling pathways and virulence.

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