The Monomeric GTPase Rab35 Regulates Phagocytic Cup Formation and Phagosomal Maturation in *Entamoeba histolytica*\(^{[5]}\)

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One of the hallmarks of amoebic colitis is the detection of *Entamoeba histolytica* (Eh) trophozoites with ingested erythrocytes. Therefore, erythrophagocytosis is traditionally considered as one of the most important criteria to identify the pathogenic behavior of the amoebic trophozoites. Phagocytosis is an essential process for the proliferation and virulence of this parasite. Phagocytic cargo, upon internalization, follows a defined trafficking route to amoebic lysosomal degradation machinery. Here, we demonstrated the role of EhRab35 in the early and late phases of erythrophagocytosis by the amoeba. EhRab35 showed large vacuolar as well as punctate vesicular localization. The spatiotemporal dynamics of vacuolar EhRab35 and its exchange with soluble cytosolic pool were monitored by fluorescence recovery after photobleaching experiments. Using extensive microscopy and biochemical methods, we demonstrated that upon incubation with RBCs EhRab35 is recruited to the site of phagocytic cups as well as to the nascent phagosomes that harbor Gal/GalNAc lectin and actin. Overexpression of a dominant negative mutant of EhRab35 reduced phagocytic cup formation and thereby reduced RBC internalization, suggesting a potential role of the Rab GTPase in the cup formation. Furthermore, we also performed a phagosomal maturation assay and observed that the activated form of EhRab35 significantly increased the rate of RBC degradation. Interestingly, this mutant also significantly enhanced the number of acidic compartments in the trophozoites. Taken together, our results suggest that EhRab35 is involved in the initial stage of phagocytosis as well as in the phagolysosomal biogenesis in *E. histolytica* and thus contributes to the pathogenicity of the parasite.

**Entamoeba histolytica** is an intestinal protozoan parasite and the causative agent of invasive amebiasis. *E. histolytica* infects around 50 million people worldwide, and approximately 100,000 die annually, making it the third common cause of death from parasitic diseases after malaria and schistosomiasis (1, 2). The infections occur in Central America, western South America, western and southern Africa, and the Indian subcontinent. The highest estimated prevalence of the infections is mainly in developing countries. The infection has a variable outcome: most of the asymptomatic and around 20% of cases develop intestinal amebiasis, which is characterized by colonic mucosa invasion and tissue destruction.

*E. histolytica* trophozoites are known to phagocytose a variety of host cells, such as erythrocytes, immune cells, apoptotic cells, and microbiota during infection. Invasion by *E. histolytica* is strongly correlated with the capacity of the parasite to kill and phagocytose host cells and erythrocytes (3, 4). Phagocytosis-deficient mutants of *E. histolytica* display reduced pathogenicity *in vitro* and *in vivo* (5, 6). Moreover, a noninvasive *Entamoeba* species, *Entamoeba dispar*, displays lower rates of phagocytosis (7, 8).

Phagocytosis, engulfment of particles usually ≥0.5 μm, is a highly evolutionarily conserved process utilized by eukaryotes. The phagocytic process begins with particle recognition and cell surface binding (9) to a specific membrane receptor that triggers a signal transduction event, leading to the reorganization of the cytoskeleton components (10) and resulting in formation of the phagocytic cup and then a nascent membrane-bound compartment, the phagosome (11). The phagosome progressively matures and eventually fuses with lysosomes and forms phagolysosomes where the ingested material is ultimately degraded (12).

Erythrophagocytosis is traditionally considered as one of the most important criteria to identify the pathogenic behavior of the amoebic trophozoites (13). Phagocytosis of the erythrocytes appears to be a receptor-mediated process (14), and evidently the amoebic Gal/GalNAc lectin acts as the receptor for the RBCs (15, 16). Over the last few years, a tremendous amount of work has been carried out to understand the molecular mechanism of the formation of the phagocytic cup that has deciphered the early signaling events occurring upon RBC internalization in *E. histolytica* (17–22).

Rab GTPases play an indispensable role in the regulation of intracellular transport, including the budding, tethering, and fusion of vesicles (23–25). Rab proteins act as molecular...
Rab35 Participates in E. histolytica Phagocytosis

switches. They alternate between an active (GTP-bound) state and an inactive (GDP-bound) state and serve as scaffolds to integrate both membrane trafficking and intracellular signaling in a temporally and spatially sensitive manner (26). The E. histolytica genome encodes a large number (>90) of Rab GTPases, a repertoire significantly higher than that of the mammalian counterpart (27). Only for a few Rab have the contributions toward the intracellular trafficking process been experimentally investigated. EhRab5 function is important for the biogenesis of prephagosomal vacuoles (PPVs) and RBC phagocytosis (28). PPVs are EhRab5- and EhRab7A-positive transient compartments that form when the parasite comes in contact with an RBC. During a very early stage of phagocytosis, EhRab5 dissociates from PPVs, and EhRab7A-positive PPVs fuse with the phagosome. Finally, EhRab7A is dissociated from the phagosome. Recently, we demonstrated that EhRab7A is important for both early and late stages of bacterial phagocytosis (29). Additionally, EhRabA (30), EhRabB (31), and EhRab8A (32) were also implicated in phagocytosis. In a recent microarray-based study, it has been shown that two amoebic Rab GTPases, EHI_146510 and EHI_164900, are specifically up-regulated upon contact of the parasite (strain HM1:IMSS) with human colon explants (82), suggesting their role in the pathogenesis. Among these two members, EHI_146510 is a homologue of human Rab35, which plays a pivotal role in phagocytosis. As phagocytosis is an essential process for survival and virulence of the parasite, detailed studies on the amoebic Rab35 will bring new insights to our understanding of the pathogenesis of this enteric protozoan. Here, using extensive microscopy, flow cytometry, biochemical, and functional assays, we demonstrated the Rab35 homologue from E. histolytica is directly involved in RBC phagocytosis via phagocytic cup formation and phagosomal maturation.

Results

EhRab35 Resides on the Large Vacuoles as Well as Smaller Vesicles and Undergoes Fast Exchange with the Cytosolic Pool—Rab35 in mammalian cells is mainly localized on the plasma membrane as well as in the cytosol and endosomal membranes (33, 34). To study its subcellular localization in amoebic trophozoites, we used an amoebic expression plasmid for EhRab35 with an N-terminal HA epitope. Amoebic cysteine synthase promoter has been widely used for many of the Rab GTPase promoters (33, 34). To examine the dynamics of EhRab35 vacuoles in amoebic transgenic trophozoites (see “Experimental Procedures” for details). After bleaching the ROI of vacuoles (diameter, 2.6 μm) by laser fluorescence, recovery in the vacuoles was monitored over time (Fig. 1F and supplemental Movie S3). FRAP measurement after photobleaching vacuoles (diameter, 2.6 μm) of EhRab35 shows rapid recovery with a mean t1/2 of 3.51 ± 0.36 s (Fig. 1F).

Therefore, our data suggest that EhRab35 resides on cytoplasmic smaller vesicles as well as larger vacuoles that are distinct from GEVs and undergo constant exchange with the cytosolic pool. These latter vacuoles are predominantly acidic in nature.

EhRab35 Is Necessary for the Early Phase of Erythrophagocytosis by E. histolytica—In previous reports, human Rab35 has been shown to regulate various cellular processes (33, 34, 41–43) in mammalian cells, including Fcγ receptor-mediated phagocytosis (44). Phagocytosis has been suggested as a marker of pathogenicity and virulence in E. histolytica. Therefore, we

The abbreviations used are: Eh, E. histolytica; FRAP, fluorescence recovery after photobleaching; PPV, prephagosomal vacuole; GEV, giant endocytic vacuole; ROI, region of interest; gs, gene-silenced; HGL, heavy subunit of Gal/GalNAC lectin; GEF, guanine exchange factor; GAP, GTPase-activating protein; PAK, p21-activating kinase; NA, numerical aperture.
investigated the role of EhRab35 in RBC phagocytosis. The EhRab35-expressing transgenic trophozoites were allowed to internalize RBCs for 10 min at 37 °C before fixation in 4% paraformaldehyde. Images acquired by laser-scanning confocal microscopy clearly showed that HA-EhRab35 is localized to the RBC-containing phagosomes (Fig. 2A). Furthermore, we sought to investigate the functional role of EhRab35 toward erythrophagocytosis in *E. histolytica*. We used EhRab35Q67L (a constitutively active GTP hydrolysis-defective mutant) and EhRab35S22N (a constitutively inactive GTP binding-defective mutant). These mutants are widely used to study the involvement of Ras superfamily proteins in intracellular signaling and vesicular trafficking. We generated stable transgenic trophozoites that expressed Q67L and S22N mutant forms of EhRab35. Expression and localization of EhRab35Q67L and EhRab35S22N mutants were confirmed by Western blotting and laser-scanning confocal microscopy (Fig. 2, B and C). Previously, we noticed that the GTP-binding defective mutant of the amoebic Rab proteins showed a low level of expression compared with wild type (WT) Rab protein in trophozoites (29). Similarly, EhRab35S22N-expressing trophozoites showed a low level of expression of the mutant. Although EhRab35S22N trophozoites showed cytosolic localization, EhRab35Q67L-expressing trophozoites showed similar subcellular localization as the WT. WT, S22N, and Q67L trophozoites were incubated with CellTracker Orange-labeled RBCs for 7.5
and 15 min at 37 °C and subsequently analyzed by flow cytometry to measure the intracellular RBCs as described earlier (37). As shown in Fig. 2D and supplemental Fig. S1, RBC uptake is significantly reduced approximately 16.1 ± 5.9 and 18.4 ± 7.1% in both the 7.5- and 15-min time points, respectively, in the EhRab35S22N-expressing trophozoites compared with the WT-expressing trophozoites. We also established the G3 strain of *E. histolytica* in which EhRab35 was gene-silenced (gs) as described earlier (45).

We observed a 63% reduction in the expression of EhRab35 in the gs strain compared with the vector control (psAP-2-Gunma)-transfected G3 trophozoite (supplemental Experimental Procedures and supplemental Fig. S2). CellTracker-labeled RBCs were used to measure the phagocytic efficiency in EhRab35-silenced and vector control trophozoites using flow cytometry. As shown in Fig. 2E, silencing of EhRab35 significantly reduced RBCs uptake (40.3 ± 5.6%).

Our functional data suggested that EhRab35 is likely to be involved in the early phase of amoebic erythrophagocytosis. Therefore, we examined the colocalization of EhRab35 with a known early phagocyte marker, such as Gal/GalNAc and actin. EhRab35WT-expressing trophozoites were incubated with RBCs for 5 min at 37 °C. Postincubation, trophozoites were fixed and immunostained for heavy subunit of Gal/GalNAc lectin (HGL), actin, and HA-EhRab35. Our microscopy results revealed that EhRab35 associated with Gal/GalNAc lectin- and actin-positive phagosomes (Fig. 2F). Furthermore, we also purified the phagosomes by sucrose gradient ultracentrifugation as described earlier (46–48) and investigated the biochemical association of EhRab35 and HGL (supplemental Fig. S3). These data suggest that the GTP-bound form of EhRab35 might be involved in the early phase of erythrophagocytosis.

**EhRab35 Localizes to Phagocytic Cup during Erythrophagocytosis**—Because the initial phase of RBC phagocytosis involves association of RBCs with amoebic phagocytic cups, we further asked whether EhRab35 is part of the phagocytic cup. EhRab35-expressing trophozoites were incubated with RBCs and fixed after 5 min. Subsequently, fixed trophozoites were immunostained for EhRab35 and actin. We observed that EhRab35 and actin are colocalized at the site of the phagocytic cups during erythrophagocytosis (Fig. 3A). Similar experiments were performed with EhRab35Q67L and EhRab35S22N mutants. The EhRab35Q67L mutant was localized to the phagocytic cups during erythrophagocytosis (Fig. 3A). Unlike wild type and the Q67L mutant, S22N mutant did not accumulate at the phagocytic cups (Fig. 3A). The active form of EhRab35 appeared to be colocalized with actin at the phagocytic cup (as shown by the arrowhead) as confirmed by the Pearson correlation coefficient (r). A quantitative comparison revealed that both the wild type and the GTP-bound form of EhRab35 showed colocalization with actin at the phagocytic cup (Fig. 3B), suggesting that the active population of EhRab35 is recruited at the site of cup initiation.

We further examined the localization of EhRab35 during erythrophagocytosis in live trophozoites. GFP-tagged EhRab35-expressing trophozoites were incubated with RBCs. Live cell imaging data revealed that GFP-EhRab35 rapidly translocated to the newly forming phagocytic cup within 4 s from the attachment of RBCs (Fig. 3C and supplemental Movie S4).

Furthermore, we quantified the number of phagocytic cups in the wild type- and mutant-expressing trophozoites. Transgenic trophozoites were incubated with RBCs for 5 min at 37 °C, and the number of phagocytic cups was determined using confocal microscopy (Fig. 4A). EhRab35S22N mutant significantly decreased the formation of phagocytic cups (Fig. 4B). To study the surface topology at high resolution, we carried out scanning electron microscopy on the WT and S22N trophozoites. After 5 min of incubation, RBCs were associated with the surface of the amoebic trophozoites expressing EhRab35WT or EhRab35S22N, and membrane extensions were observed around the RBCs. EhRab35WT-expressing trophozoites were spread and showed extensive membrane around the RBCs (Fig. 4C). In EhRab35S22N trophozoites, the pseudopods did not

**FIGURE 1. Cellular distribution and cyclic exchange dynamics of EhRab35.** A, cellular localization of HA-EhRab35 in *E. histolytica*. Amoebic trophozoites were transfected with the plasmid pEHEX-HA (control) or with the construct pEHEX-HA-EhRab35 and incubated on glass slide for 15 min at 37 °C in the steady-state condition. Cells were then fixed and processed for immunofluorescence using anti-HA antibody. White arrowheads indicate the large vacuolar compartments, and yellow arrowheads show small cytoplasmic vesicles. B, cell lysates from plasmid pEHEX-HA (control) and HA-EhRab35-expressing trophozoites (100 μg) were resolved by SDS-PAGE and subjected to immunoblotting using anti-HA antibody. White arrowheads indicate the large vacuolar compartments, and yellow arrowheads show small cytoplasmic vesicles. C, live amoebic trophozoites expressing GFP-EhRab35. Amoebic transformants expressing GFP-tagged plasmid and GFP-EhRab35 trophozoites were incubated on MatTek Corp. glass bottom dishes for 15 min at 37 °C, and the number of phagocytic cups was determined using confocal microscopy (Fig. 4A). EhRab35S22N mutant significantly decreased the formation of phagocytic cups (Fig. 4B). To study the surface topology at high resolution, we carried out scanning electron microscopy on the WT and S22N trophozoites. After 5 min of incubation, RBCs were associated with the surface of the amoebic trophozoites expressing EhRab35WT or EhRab35S22N, and membrane extensions were observed around the RBCs. EhRab35WT-expressing trophozoites were spread and showed extensive membrane around the RBCs (Fig. 4C). In EhRab35S22N trophozoites, the pseudopods did not
extend all around the RBCs bound to the trophozoite surface (Fig. 4C).

In addition, EhRab35gs trophozoites were used to probe the role of EhRab35 in phagocytic cup formation. Our results suggest that phagocytic cup formation is significantly affected in the EhRab35gs trophozoites compared with that in the vector control trophozoites (Fig. 4, D and E). Taken together, these data show that EhRab35 is recruited to the phagocytic cup during RBC internalization and thereby likely play an important role in the actin cup formation.
EhRab35 Facilitates RBC Degradation via Induction of Amoebic Lysosome-like Compartments—As evident from the fixed and live cell imaging data (Fig. 1H and supplemental Movie S2), EhRab35 is localized to the acidic vacuolar compartments. These results prompted us to investigate whether EhRab35 also plays any role in phagosomal maturation. Here, we carried out a microscopy-based assay to quantify RBC degradation. Transgenic trophozoites expressing wild-type or mutant EhRab35 were pulsed with RBCs for 5 min at 37 °C. Cells were then fixed and processed for immunofluorescence using anti-HA and phalloidin. Green arrowheads indicate EhRab35, and red arrowheads indicate actin localization at the phagocytic cup. White arrowheads indicate the colocalization, and the white arrow shows no association between EhRab35 and actin. B, colocalization between EhRab35 and actin at phagocytic cup. Colocalization was quantified using the ImageJ-Fiji software to obtain Pearson’s correlation coefficient (r). The quantification was performed on an ROI at the site of the phagocytic cup in 15 independent cells for each transgenic line from three independent experiments. Data points shown in the bar graph represent means and S.D. (error bars). C, confocal live cell imaging of EhRab35 in E. histolytica trophozoites during the phagocytosis of RBCs. Amoebic stable transformants expressing GFP-EhRab35 were allowed contact with RBCs and observed by confocal microscopy. Binding of RBCs to the amoebic trophozoite is set as time 0 (seconds). Phagocytic cup initiation is marked by the white arrowheads, the white arrow indicates the forming phagosome, and red asterisks indicate RBCs. The corresponding movie is supplemental Movie S4. All scale bars, 10 μm. DIC, differential interference contrast.

FIGURE 3. EhRab35 is part of phagocytic cup. A, a GTP-bound form of EhRab35 colocalized with actin during erythrophagocytosis. Amoebic trophozoites stably expressing EhRab35WT, EhRab35Q67L, or EhRab35S22N were pulsed with RBCs for 5 min at 37 °C. Cells were then fixed and processed for immunofluorescence using anti-HA and phalloidin. Green arrowheads indicate EhRab35, and red arrowheads indicate actin localization at the phagocytic cup. White arrowheads indicate the colocalization, and the white arrow shows no association between EhRab35S22N and actin. B, colocalization between EhRab35 and actin at phagocytic cup. Colocalization was quantified using the ImageJ-Fiji software to obtain Pearson’s correlation coefficient (r). The quantification was performed on an ROI at the site of the phagocytic cup in 15 independent cells for each transgenic line from three independent experiments. Data points shown in the bar graph represent means and S.D. (error bars). C, confocal live cell imaging of EhRab35 in E. histolytica trophozoites during the phagocytosis of RBCs. Amoebic stable transformants expressing GFP-EhRab35 were allowed contact with RBCs and observed by confocal microscopy. Binding of RBCs to the amoebic trophozoite is set as time 0 (seconds). Phagocytic cup initiation is marked by the white arrowheads, the white arrow indicates the forming phagosome, and red asterisks indicate RBCs. The corresponding movie is supplemental Movie S4. All scale bars, 10 μm. DIC, differential interference contrast.

EhRab35 Participates in E. histolytica Phagocytosis

Rab35 Participates in E. histolytica Phagocytosis

MARCH 24, 2017 • VOLUME 292 • NUMBER 12

JOURNAL OF BIOLOGICAL CHEMISTRY

4965
tion, we used LysoTracker fluorescence as an indicator of gross differences in acidity among populations. We used EhRab35WT, EhRab35Q67L, and EhRab35S22N mutants for LysoTracker staining. LysoTracker-labeled trophozoites were analyzed by flow cytometry as described earlier (35, 39). Flow cytometric analysis showed a significant increase of the LysoTracker content in the EhRab35Q67L-overexpressing transformants compared with the control transformants (Fig. 5B). Similar experiments were performed with all the transgenic lines using confocal microscopy. Quantification of the microscopy data revealed that the EhRab35Q67L-containing vacuoles are predominately positive for LysoTracker (Fig. 5, C and D).

In addition, we performed ammonium chloride (NH₄Cl) pulse experiments on the trophozoites. NH₄Cl is a weak base that acts as a lysosomal inhibitor by reducing the acidification of lysosome. It is often used in functional studies on lysosomes (50). Treatment with NH₄Cl raises lysosomal pH (51), which results in the diffusion of LysoTracker dye from the acidic compartments. Untransfected amoebic trophozoites were incubated with 10 mM NH₄Cl for 10 min. NH₄Cl-treated trophozoites showed a drastic reduction in LysoTracker fluorescence (Fig. 5, E and F). Similar experiments were performed with WT and mutant EhRab35 transgenic trophozoites. As expected, relative LysoTracker fluorescence was reduced in the transgenic trophozoites (Fig. 5, G and H) upon NH₄Cl treatment. Surprisingly, the effect of the treatment was minimal for the wild type trophozoites. Accordingly, the immunofluorescence experiments revealed that the wild type EhRab35 trophozoites could maintain a significant population of acidic compartments after the treatment (supplemental Fig. S5). The above results thus suggest that overexpression of EhRab35Q67L enhances relatively acidic compartments in the trophozoites. It also indicates that the GTPase activity of the protein is important to maintain the integrity of these compartments under weak base condi-
tions. To further demonstrate that these acidic compartments contribute to the phagolysosomal maturation of ingested erythrocytes, we performed an erythrophagocytosis assay using a pulse-chase approach. HA-tagged EhRab35 and its mutant transgenic trophozoites were stained with phalloidin. Confocal images were acquired, and the number of actin phagocytic cups were counted in each transfectant. A total of 100 cells were used for each condition from three independent experiments. Data points shown in the bar graph represent means and S.D. Statistical significance was determined using unpaired two-tailed Student’s t test. ***, p < 0.001. C, morphology of EhRab35 transgenic trophozoites visualized by scanning electron microscopy. Stably expressing WT and EhRab35S22N amoebic trophozoites were pulsed with RBCs for 5 min and visualized. The red arrowheads show an attached RBC. D, psAP-2-Gunma and psAP-2-GunmaEhRab35gs trophozoites were incubated with RBCs for 5 min at 37 °C. Then they were stained with phalloidin. Confocal images were acquired, and the total number of actin phagocytic cups was counted in each transgenic line. E, quantitative estimation of total phagocytic cup formation. A total of 540 (psAP-2-Gunma) and 489 (psAP-2-GunmaEhRab35gs) trophozoites were used from three independent experiments. The bar graph represents the means and S.D. (error bars). Data are normalized by considering the psAP-2-Gunma (vector control) as 100%. Statistical significance was determined using unpaired two-tailed Student’s t test. **, p < 0.01. All scale bars, 10 μm. DIC, differential interference contrast; Mag, magnification; WD, width; EHT, extra high tension.
EhRab35 in a later stage of RBC phagocytosis in live amoeba, GFP-EhRab35-expressing transgenic trophozoites were incubated with RBCs for 15 min, chased for 120 min at 37 °C, and visualized using confocal microscopy. Our live cell imaging data revealed that EhRab35 is associated with RBC-containing vacuoles (Fig. 6 and supplemental Movie S5). Collectively, our results indicate that EhRab35 compartments are potentially involved in phagosome maturation in *E. histolytica*.
Rab35 Participates in *E. histolytica* Phagocytosis

The current study demonstrates the contribution of the EhRab35 toward phagocytosis of human erythrocytes by the protozoan parasite *E. histolytica*. Here, using paraformaldehyde-fixed and live amoebic trophozoites, we have studied the subcellular localization of Rab35 orthologue during phagocytosis of human erythrocytes by *E. histolytica*. A functionally similar pathway may also exist in other species.

Rab GTPases are molecular switches, and they play a central role in intracellular membrane trafficking and compartmental-
Rab35 Participates in *E. histolytica* Phagocytosis

Rab proteins are involved in diverse processes, such as protein secretion, receptor recycling, vesicle fission and fusion, and lipid distribution (25, 53). The mammalian Rab1 subfamily consists of Rab1A, Rab1B, and Rab1C (also named Rab35). Rab1A and Rab1B have been implicated in endoplasmic reticulum to Golgi transport (54, 55). The function of Rab35 has mainly been explored in *Drosophila melanogaster* and mammalian cell lines (34, 56). Recently, Rab35 has also been shown to be associated with cancer (41). Moreover, it has been shown to be linked to the secretion of exosomes (57) and fast recycling of transferrin (58) and major histocompatibility complex class II (MHCII) (59) to the plasma membrane. Its role in postfurrowing cytokinesis events has also been demonstrated in a separate study (33). Cells with depleted levels of Rab35 or transferrin receptor showed significantly lower intracellular iron levels and reduced ability to support uropathogenic *Escherichia coli* survival in the mammalian system (43). Thus, mammalian Rab35 is a multifaceted protein, playing crucial roles in various pathophysiological processes.

Amoebic EhRab35 is localized to both large vacuolar and smaller punctate structures that are distinct from giant early endocytic vacuoles (38). We also developed FRAP-based methodology to study the exchange of EhRab35 between the vacuolar compartment and cytosolic pool. A similar study has also been reported in HeLa cells (60). Interestingly, EhRab35 showed much faster recovery than its human homologue. Rab GTPases are known to cycle between the cytosol (GDP-bound) and the membrane (GTP-bound). The GDP-GTP cycle is regulated by guanine exchange factors (GEFs) and GTPase-activating proteins (GAPs). Although GEFs are involved in activating the Rabs, GAPs bring them back to the inactivated state by catalyzing GTP hydrolysis. The effective rate of partitioning of a Rab GTPase from cytosolic to membrane compartments is thus determined by the combined activity of these regulators. Thus, the current experimental setup could be useful to design an in vivo assay for quantifying GEF and GAP activity in the parasite. Rab GTPases direct membrane targeting during phagocytosis. Rab5 enables fusion of the early endosomes to the nascent phagosomes and further helps in the recruitment of Rab7, which in turn promotes the formation of phagolysosomes in mammalian cells (61–63). In Fcy receptor-mediated phagocytosis, both Rab5 (64) and Rab35 (44) have been shown to be involved in the early stage. In the amoebic trophozoites, EhRab5 and EhRab7A have been shown to play an important role in the intracellular events during erythropagocytosis. In particular, whereas EhRab5 is involved in the biogenesis of PPVs and accelerates RBC engulfment by amoeba (28), EhRab7A contributes to a later stage of phagocytosis (28). EhRab35 is involved in the early stage of phagocytosis. The EhRab35S22N trophozoites showed a marginal reduction in RBC uptake. Notably, EhRab35gs trophozoites significantly reduced the uptake. The mild effect on RBC uptake was observed in S22N trophozoites. This might be due to insufficient competition of the poorly expressing mutant protein with the endogenous EhRab35. It has been reported in the literature that the dominant negative form of Rab proteins could sometimes be unstable and undergo degradation, thus accounting for the subtle cellular phenotype (65).

The signaling events that occur during different stages of phagocytosis have been partially characterized in *E. histolytica*. Remodeling of the actin cytoskeleton is an essential step during the early stage of phagocytosis. In mammalian cells, this process is mainly regulated by the Rho family of small GTPases and their downstream effectors. In the amoebic parasite, only a few such molecular components, such as EhCdc42 and its effectors EhPAK and EhRac1, have been characterized (66). Interestingly, during the initial stage of erythropagocytosis, Ca$^{2+}$ signaling (18) plays an important role in the recruitment of actin (21). In this context, the roles of some of the amoebic calcium-binding proteins, including EhCaBP1 (19) and EhCaBP3 (20), have been studied wherein these proteins were shown to recruit the amoebic homologue of Arp2/3 complex as well as amoebic myosin via recruitment of EhAK1 at the site of erythrocyte internalization (21, 22, 67, 68).

Human Rab35 localizes to the plasma membrane (33) and continues to the early and late phagosomes (44, 69). In this study, we observed that EhRab35 is recruited to the site of pseudopod extension, which precedes phagocytic cup formation. We hypothesize that incubation with the RBCs triggers very early signaling events in the amoeba that result in the recruitment of the Rab GTPase to the phagocytic cups. The recruitment could possibly be driven by two distinct mechanisms. A spatiotemporal activation of a GEF for EhRab35 could in turn activate the GTPase at the site of cup formation. Alternatively, the signaling event could trigger fusion of the EhRab35-containing vacuoles with the plasma membrane at the site of cup formation. Both mechanisms could also contribute to the observed translocation of the GTPase. Currently, it is not known whether the recruitment of EhRab35 is upstream or downstream of the recruitment of actin to the phagocytic cup. Interestingly, some of the actin remodeling molecules, such as Rab35, Arp2/3, PAK1, and Cdc42, are functionally conserved from mammals to the amoebic parasite (22, 44, 66, 70–72). However, the molecular mechanism linking the Rab35 to Arp2/3, PAK1, and Cdc42 has not been established in mammalian cells or *E. histolytica*.

Rab7 is a well established marker for the late endosome and is implicated in lysosome biogenesis and phagosomal maturation in mammalian cells (61, 73, 74). Rab35 is implicated in the fusion of phagosomes and lysosomes during the process of phagosome maturation (69). In the current study, we found that the overexpression of Q67L mutant of EhRab35 caused a significant increase in the number of LysoTracker-positive compartments. However, EhRab35S22N mutant trophozoites did not show any significant effect on LysoTracker-positive compartments. This perhaps suggests that the GDP-bound form of EhRab35 acts as an enhancer for the biogenesis of lysosome-like compartments without playing any essential role. Furthermore, the overexpression of EhRab35Q67L mutant trophozoites significantly enhances RBC degradation, which corroborates well with the accumulation of acid hydrolases in this Rab compartment. An alternative mechanism of Rab-assisted degradation of phagosomal cargoes has already been reported in the literature: the amoebic trophozoites utilize EhRab7A-positive PPVs to acidify the phagosomal compartments gradually via fusion.
Rab35 Participates in E. histolytica Phagocytosis

amoebic expression plasmid, sequenced, and hypothetically translated into a 199-amino acid sequence. The sequence analysis of the cloned protein (EhRab35) revealed the presence of highly conserved motifs of Rab proteins, such as the guanine nucleotide binding regions, effector loop, and C-terminal iso-prenylation motif (supplemental Fig. S6). Comparison of the EhRab35 sequence by Clustal Omega multiple sequence alignment demonstrated that the cloned protein has a similarity of 55% with the H. sapiens Rab35 sequence.

Cloning and Plasmid Construction—To clone Rab35 from Entamoeba, a putative Rab35 sequence was identified from the E. histolytica genome with substantial homology with human Rab35 using BLAST. Forward (5′-GCACCCGGATGGCA-CAAGAATTGATTATTTTT3′) and reverse (5′-GACCTCGAGTAAACACATCTCGTGTTAGGTTCTTTTG-3′) primers were designed, and PCR was performed using the cDNA pool. The amplified PCR fragment was subjected to restriction digestion with SamI and XhoI and ligated into pEhEx/HA and pEhEx/GFP using T4 ligase (New England Biolabs).

For the generation of the EhRab35-silencing strain, the following primer set was used for PCR amplification: forward, 5′-GCAAGGCTATGATTTGTGATTACGTTGGA- AAAG-3′; and reverse, 5′-GACGAGCTCCATTCCTCTC- TTGGTTTTGAAATTCCTGTCG-3′. A 400-bp segment of EhRab35 was amplified. The amplified PCR fragment was further cloned into psAP-2-Gunma using Stul/Sacl to generate the gene-silencing plasmid psAP-2-GunmaEhRab35.

Overexpression of EhRab35 and Its Mutants in E. histolytica—To overexpress EhRab35 or its mutants in E. histolytica as an HA or GFP fusion protein with a HA/GFP tag at the N terminus, the full-length EhRab35 or its mutant gene was transfected by electroporation as described earlier (76). Briefly, Entamoeba trophozoites were collected from the log phase cultures and washed with PBS followed by incomplete cytokin buffer (10 mM K2HPO4/KH2PO4 (pH 7.6), 120 mM KCl, 0.15 mM CaCl2, 25 mM HEPES (pH 7.4), 2 mM EGTA, 5 mM MgCl2). The washed cells were then resuspended in 0.4 ml of complete cytokin buffer (incomplete cytokin containing 4 mM adenosine triphosphate and 10 mM reduced glutathione) containing 100 μg of plasmid DNA and subjected to consecutive pulses of 500-V voltage and 500-μF capacitance (Bio-Rad Gene Pulser M Xcell) to facilitate DNA uptake by trophozoites. Electroporated trophozoites were then transferred into a drug-free medium for 48 h at 35.5 °C. Subsequently, stable clones were selected in the presence of 4 μg/ml G418 (catalogue number 1720, Sigma-Aldrich). All the experiments were performed at 20 μg/ml G418.

Overexpression of the respective protein was confirmed by Western blotting analysis using the indicated antibodies and by confocal microscopy.

For generating the EhRab35-silencing strain, G3 trophozoites were electroporated with psAP-2-GunmaEhRab35 and psAP-2-Gunma as described above. A similar procedure was used for selecting the transgenic EhRab35-silencing G3 trophozoites.

RBC Labeling—Ethical clearance from the Institute Ethical Committee, Indian Institute of Science Education and Research, Bhopal, India was obtained. The human RBCs used in

Experimental Procedures

Culture of E. histolytica Trophozoites—E. histolytica strain HM1:IMSS cl6 and G3 trophozoites were grown axenically in BI-S-33 medium supplemented with 15% (v/v) heat-inactivated adult bovine serum (catalogue number B-9433, Sigma-Aldrich), 100 units of penicillin/ml, and 100 μg streptomycin sulfate/ml (Invitrogen) at 35.5 °C as described previously (75).

In Silico Identification of Rab35 Homologue from E. histolytica—Rab35 protein belongs to the Rab1 subfamily. To clone a Rab35 homologue from E. histolytica, a BLAST search was performed on amoeba database (AmoebaDB) using the Homo sapiens Rab35 sequence as a query. A putative Rab1B (EHL_146510) and a putative Rab1A sequence (EHL_108610) from the E. histolytica genome showing 55 and 48% identity, respectively, to the human Rab35 sequence were recognized. The putative Rab1B GTPase sequence showed highest (55%) identity with human Rab35, giving strong support to the annotation of EhRab1B as EhRab35. Furthermore, using forward primer and reverse primer, we amplified a 600-bp fragment from the E. histolytica cDNA pool by PCR. The PCR product was cloned in the

FIGURE 7. EhRab35 regulates phagocytic cup formation and phagosomal maturation in E. histolytica. EhRab35 localized on acidic vacuoles. A small population of the GTPase is also associated with EhRab7A- and transferrin-containing GEVs (38). The GTPase cycle of EhRab35 is important for maintaining the integrity of the acidic vacuoles in the presence of NH4Cl. Interestingly, upon incubation of trophozoites with RBCs, EhRab35 translocates from the cytosol to the phagocytic cup and further remains associated with the phagolysosome. The time scale shown in this model for RBC phagocytosis is obtained from the current study.

with amoebic lysosome-like compartments, which harbor digestive hydrolases, cysteine proteinases, and membrane-permeabilizing peptides (28). In summary, we observed that EhRab35 is recruited to the site of a phagocytic cup and continues to associate with the late or lysosomal compartments during phagocytosis (Fig. 7). Further identification of the upstream regulators and effectors of the Rab GTPase will provide molecular insights into the role of the protein in this complex process. Taken altogether, this study provides direct evidence that EhRab35 might help the parasite during the host tissue invasion (82).
all the experiments were collected from Kuldeep Verma. RBCs were briefly washed with 1% bovine serum albumin (BSA) (in 1× PBS) and immediately incubated with CellTracker Orange (50 μg/ml) or Blue (20 μl) (catalogue numbers C2927 and C2110, Life Technologies) for 45 min at 37 °C in the dark. Afterward, the RBCs were subsequently washed four times with 1% BSA. RBC labeling was confirmed by fluorescence microscopy, and the RBCs were suspended in the BI-S-33 medium for experiments.

**Immunofluorescence Assay**—The immunofluorescence assay was performed as described previously (38). Trophozoites were incubated for 1 h in a 1:20 dilution of primary antibodies rabbit polyclonal anti-EhRab7A, mouse monoclonal anti-HA (catalogue number sc-7392, Santa Cruz Biotechnology), and anti-HGL (1:100) at room temperature. After three washes in blocking solution, trophozoites were co-incubated with Alexa Fluor-conjugated (Life Technologies) secondary antibodies (1:500 dilutions) and Alexa Fluor 568-phalloidin (1:50 dilutions) for 1 h at room temperature. After three washes with blocking solution, coverslips were mounted on the glass slide using Mowiol. Slides were examined using a LSM-780 laser-scanning confocal microscope (Carl Zeiss, GmbH, Jena, Germany) with a 63×/1.4 NA oil immersion objective lens. Immunofluorescence signals were captured in individual en face (xy axes) planes throughout the cellular z axis at 0.35-μm intervals.

**Live Cell Imaging**—The trophozoites expressing GFP-tagged fusion proteins were grown in 20 μg/ml G418, harvested in log phase, washed, and resuspended in BI-S-33 medium. Trophozoites were transferred to glass bottom dishes (MatTek Corp.), allowed to settle for 10 min, and observed using a Leica TCS SP8 inverted confocal microscope equipped with a Leica motCORR™ Plan Apochromatic 63×/1.4 NA oil immersion objective inverted microscope (Leica Microsystems, Germany). An argon laser (488 nm) was used for excitation of GFP. At least five examples were observed in each experiment, and one representative video is shown.

**FRAP Analysis**—FRAP was performed using the FRAP module on a Leica TCS SP8 confocal microscope. For FRAP, the amoebic trophozoites harboring GFP-EhRab35 were observed 24 h after addition of 20 μg/ml G418 using a Leica TCS SP8 inverted confocal microscope equipped with a Leica motCORR Plan Apochromatic 63×/1.4 NA oil immersion objective and a 488 nm argon laser. Bleaching was performed during fly forward using ROI scan features and high laser power. In the FRAP experiments, spherical areas of approximately >5-μm diameter were photobleached for 1 s, and subsequently images of the area were collected every 5.3 frames/s. To calculate the time of fluorescence signals were captured in individual en face (xy axes) planes throughout the cellular z axis at 0.35-μm intervals.

**NH₄Cl Pulse Assay**—LysoTracker Red DND-99-labeled amoebic trophozoites were seeded in a chamber slide or 24-well plate and then incubated with 10 mM NH₄Cl in regular serum.
free medium (BI-S-33 medium) for 10 min at 37 °C. After that, NH₄Cl was removed, and trophozoites were washed with BI-S-33 medium and subsequently fixed with paraformaldehyde (for microscopy). For flow cytometric analysis, NH₄Cl was removed, and trophozoites were washed with cold BI-S-33 medium and once washed with 1× PBS.

Scanning Electron Microscopy—To analyze the morphology of phagocytic cups, trophozoites were incubated with RBCs on a glass coverslip for 2 min at 37 °C. Phagocytosis was arrested by fixation in 2.5% glutaraldehyde in phosphate buffer (pH 7.4) at 4 °C overnight. Dehydration was carried out with an ascending series of ethanol solutions (25, 50, 75, and 95%) for 15 min each at room temperature followed by 100% for 15 min at room temperature three times. Finally, samples were dried at room temperature for 72 h, mounted onto stubs, and sputter-coated with a thin layer of gold with the use of a Quorum Q150R ES Sputter Coater. Images were acquired with a Zeiss (Ultra Plus) high resolution field emission scanning electron microscope (HR FESEM).

Statistical Analysis—Statistical significance was determined by the differences between treatments using unpaired two-tailed Student’s t test using the GraphPad Prism version 4.0 (GraphPad Software).

Author Contributions—K. V. and S. D. conceived and designed the experiments and wrote the paper. K. V. performed the experiments. Both authors analyzed the results and approved the final version of the manuscript.

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