Rab5-associated Vacuoles Play a Unique Role in Phagocytosis of the Enteric Protozoan Parasite Entamoeba histolytica*

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In mammals, Rab5 and Rab7 play a specific and coordinated role in a sequential process during phagosome maturation. Here, we report that Rab5 and Rab7 in the enteric protozoan parasite Entamoeba histolytica, EhRab5 and EhRab7A, are involved in steps that are distinct from those known for mammals. EhRab5 and EhRab7A were localized to independent small vesicular structures at steady state. Priming with red blood cells induced the formation of large vacuoles associated with both EhRab5 and EhRab7A (“prephagosomal vacuoles (PPV)”) in the amoeba within an incubation period of 5–10 min. PPV emerged de novo physically and distinct from phagosomes. PPV were gradually acidified and matured by fusion with lysosomes containing a digestive enzyme, cysteine proteinase, and a membrane-permeated by fusion with lysosomes containing a digestive enzyme. After EhRab5 disassociated from PPV, 5–10 min later, the EhRab7A-PPV fused with phagosomes, and EhRab7A finally dissociated from the phagosomes. Immunoelectron and light micrographs showed that PPV contained small vesicle-like structures containing fluid-phase markers and amoebapores, that were not evenly distributed within PPV, suggesting that the mechanism was similar to multivesicular body formation in PPV generation. In contrast to Rab5 from other organisms, EhRab5 was involved exclusively in phagocytosis, but not in endocytosis. Overexpression of wild-type EhRab5 enhanced phagocytosis and the transport of amoebapore to phagosomes. Conversely, expression of an EhRab5Q67L GTP form mutant impaired the formation of PPV and phagocytosis. Altogether, we propose that the amoebic Rab5 plays an important role in the formation of unique vacuoles, which is essential for engulfment of erythrocytes and important for packaging of lysosomal hydrolases, prior to the targeting to phagosomes.

Phagocytosis is a critically important element of host defense against invading pathogens in higher organisms and its molecular mechanism in professional phagocytes, e.g. macrophage, has been extensively studied at the molecular level (1, 2). A number of steps including cell surface binding to ligands and the activation of a signaling pathway leading to F-actin polymerization have been identified as essential for phagocytosis. In addition, membrane trafficking plays an important role in the controlled maturation of phagosomes. The maturation is accompanied by sequential fusion with the endocytic compartment to form a phagolysosome, and is orchestrated by small GTPases, Rab proteins, which act as molecular switches regulating the fusion of vesicles with target membranes through the conformational change between active (GTP-bound) and inactive (GDP-bound) forms (3). It has been reported that Rab5 and Rab7 play an important role in the maturation of phagosomes in macrophages (4).

Rab5 was initially shown to be localized to early endosomes and the plasma membrane, and involved in endocytosis and the endosome fusion (5, 6). Rab5 was also observed on nascent phagosomes, and has been implicated to play an important role in the fusion between phagosomes and early endosomes (7–9). Expression of the GTP form Rab5Q67L mutant or down-regulation of wild-type Rab5 by antisense oligonucleotides perturbed the regulated fusion between phagosomes and endosomes, and resulted in the formation of giant phagosomes in the former case, and reduced activity for killing of ingested bacteria because of the inhibition of phagosomal maturation in the latter case (8, 9). In addition to Rab5 per se, some of the Rab5 effectors that were implicated in endosome fusion, e.g. EEA1 and phosphatidylinositol 3-kinase (Vps34) (10, 11), also have been identified on the phagosome membrane, suggesting that phosphoinositide metabolism is important for phagosome maturation as seen in the endocytic pathway (12, 13). Rab7 has been implicated in late endosomal membrane trafficking in the endocytic pathway (14), and also in the late stage of phagosome maturation (4, 13). Although a specific role for Rab7 during phagocytosis has not yet been well demonstrated, some intracellular microorganisms have been reported to be capable of blocking the maturation and acidification of phagosomes by interfering with Rab7 (15, 16). It has also been recently demonstrated that a novel effector protein, RILP, is recruited to the phagosomal membrane by Rab7, which promotes fusion between phagosomes and lysosomes (17).

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Entamoeba Rab5 in Phagocytosis

Besides professional phagocytes from higher eukaryotes, some unicellular organisms such as *Dictyostelium discoideum* and *Entamoeba histolytica* show an inherent ability of phagocytosis. *E. histolytica*, an entero protozoan parasite that causes an estimated 50 million cases of amebiasis: amebic colitis, dysentery, and extraintestinal abscesses (18), and 40,000–100,000 deaths annually (19), colonizes the human gut and engulfs foreign cells including microorganisms and host cells. Phagocytosis has been implicated to be closely associated with the pathogenesis of the amoeba because phagocytosis-deficient amoeba mutants were shown to be avirulent (20). Although a number of amoebic molecules involved in attachment, phagocytosis, and degradation of microorganisms and host cells have been identified including galactose/N-acetylgalactosamine (Gal/GalNAc)-inhibitable lectin (21, 22), cytoskeletal proteins and their associated regulatory molecules (23–25), cysteine proteinases (CP), and pore-forming peptides (i.e. amoebapore) (26, 27), the molecular mechanism of phagocytosis in this parasite remains largely unknown.

We presumed that Rab proteins also play an essential and central role in the regulation of phagocytosis and endocytosis in *E. histolytica*. We and other groups (28–31) have reported about 20 EhRab genes. An additional 50 putative Rab genes showing significant homology to Rab from other organisms were found in the *E. histolytica* genome data base (data not shown, www.tigr.org). A few EhRab proteins have been shown to participate in phagocytosis. *EhRabB* was shown to be located on the plasma membrane and phagocytic mouths in the early phase (up to 5 min) of phagocytosis (29). Putative *EhRab7* and *EhRab11* proteins were reported to be abundant in the endosome fraction labeled with iron-dextran, similar to their putative homologues from mammals (30). To dissect the molecular mechanism of Rab proteins involved in the phagosome biogenesis in *Entamoeba*, we characterized, in the present study, two amebic Rab proteins, *EhRab5* and *EhRab7A*, that show significant homology to mammalian and yeast counterparts. The amoebic Rab5 homologue has several unique characteristics that are dissimilar to those of the mammalian and yeast Rab5. First, *EhRab5* is primarily involved in phagocytosis, not endocytosis. Second, in contrast to mammalian Rab5, which is immediately recruited to phagosomes after the engulfment of bacteria or beads, *EhRab5* is not recruited directly to phagosomes, but colocalizes with *EhRab7A*, forming prephagosomal vacuoles (PPV) prior to fusion with phagosomes. Third, *EhRab5* is required for the formation of PPV and efficient engulfment of red blood cells. Fourth, *EhRab5* plays an important role in the transport of the major membrane-permeabilizing peptide amoebapore. Therefore, in conjunction with *EhRab7A*, *EhRab5* plays a key role in the biogenesis of phagosomes by regulating the formation of PPV and transport of membranolytic and hydrolytic factors during phagocytosis in this parasite.

**EXPERIMENTAL PROCEDURES**

Organism and Culture—*E. histolytica* trophozoites of HM-1:MMSS cl 6 (32) were cultured axenically in BI-S-33 medium at 35 °C as described previously (33).

Isolation of *EhRab5* and *EhRab7A* cDNAs—A full-length *EhRab5* cDNA was obtained by a degenerate PCR approach, followed by 5′- and 3′-rapid amplification of cDNA ends as previously described (28). A full-length *EhRab7A* gene was obtained by reverse transcriptase-PCR using oligonucleotide primers designed based on sequences previously reported (30, 34). We identified at least eight genes showing significant homology to Rab7 from other species (data not shown). We designated the *EhRab7* gene showing highest homology to mammalian and yeast Rab7 as *EhRab7A* in the present study and describe the characterization of other *EhRab7* isoforms elsewhere.

**Plasmid Constructions to Produce Transgenic Ameoba Lines—** *EhRab5* and *EhRab7A* cDNA fragments were amplified by PCR using sense and antisense oligonucleotides containing appropriate restriction sites at the end. Three tandem repeats of hemaggulutinin (HA) or c-Myc tags, made of annealed complementary oligonucleotides, were inserted in the engineered Nhel site, which was located at the fourth or second codon exposed to EhRab5 and EhRab7A cDNA (Fig. 1). An expression plasmid, pEHEx, contains the 5′-flanking region of cytoseine synthase gene (AB000266) containing a putative promoter (35), BglII and Xhol sites between cytoseine synthase 5′- and 3′-flanking regions to insert a gene of interest, cytoseine synthase 3′-flanking regions and neomycin resistance gene flanked by the 5′ and 3′ regions of actin gene, obtained from pA5′A3′ NEO (36), for drug selection. The 3HA-EhRab5 cDNA fragment was inserted into the BglII-Xhol sites of pEHEx to produce pH5. EhRab5-SQ67L and EhRab5S22N mutants were constructed by PCR-mediated mutagenesis (37). Two EhRab5 mutants were fused with the 3-HA tag and cloned to pEHEx to produce pH5L or pH5N, respectively. Plasmids to co-express *EhRab5* and *EhRab7A* were constructed by PCR amplification as described above (pH5L-M7 or pH5N-M7, respectively). A plasmid to express green fluorescent protein (GFP)-EhRab5 fusion protein in amoeba was constructed. GFP was amplified by PCR from GCR222 as a template (38), and cloned into pKT-3M, which contained the cytoseine synthase promoter, 3-Myc tag, and Smal and Xhol restriction sites to produce a pKT-MG. The EhRab5 protein coding region without the stop codon was ligated into Smal-Xhol sites of pH5-MG to produce pKT-GFP. Detailed information, e.g. nucleotide number based on sequences deposited in the data base and positions of inserted restriction sites and 3-HA or 3-Myc epitope, are also shown in Fig. 1B.

Establishment of Epitope-tagged EhRab-expressing Ameoba Cell Lines—Wild-type trophozoites were transformed with plasmids by liposome-mediated transfection as previously described (39). Transformants were initially selected in the presence of 3 μg/ml of Geneticin (Invitrogen). The Geneticin concentration was gradually increased to 6–20 μg/ml during the following 2 weeks before the transformants were subjected to analyses.

**Antibodies—** Affinity purified anti-EhRab5 or anti-EhRab7A rabbit antibodies were commercially produced at Oriental Yeast Co. (Tokyo, Japan) using recombinant amino-terminal glutathione S-transferase fusion proteins purified using glutathione-Sepharose 4B (Amersham Pharmacia Biotech), Anti-HA 16C12 was from the American Type Culture Collection. Anti-Myc monoclonal antibodies were purchased from Berkshire Antibody Co. (Berkeley, CA). Alexa Fluor anti-mouse and anti-rabbit IgG were obtained from Molecular Probes (Eugene, OR). Anti-amoebic CP2 and human band 3 rabbit antibodies were gifts from Iris Bruchhaus and Egbert Tannich (40), and Yuichi Takakuwa (41), respectively. The production of anti-amoebapore A antibody was previously described (42).

**Indirect Immunofluorescence Assay—** Foci of transfectants in a logarithmic growth phase were harvested and transferred to 8-mm round wells on glass slides and incubated for 30 min at 35 °C to let trophozoites attach to the glass surface. Gerbil red blood cells were added to each well at 10° cells/ml and incubated for 5–50 min at 35 °C. An indirect immunofluorescence assay was performed as follows. Amebae were fixed with 3.7% paraformaldehyde in phosphate-buffered saline (PBS) for 10 min at room temperature. Fixed red blood cells were stained with diaminobenzidine (0.84 mM 3,3′-diaminobenzidine, 0.048% H2O2, and 50 mM Tris-HCl, pH 9.5) for 5 min (43). Cells were then permeabilized with 0.05% Triton X-100, PBS for 5 min. Samples were reacted with 16B12 (1:1000), 9E10 (1:400), anti-amoebapore A antibody (1:1000), or affinity-purified anti-EhRab5, anti-EhRab7A, or CP2 antibody (1:200). In most experiments, we used a rabbit antibody raised against recombinant EhRab5, amoebapore, and CP, and an anti-Myc monoclonal antibody to detect 3Myce-EhRab7A unless mentioned otherwise. The samples were then reacted with Alexa Fluor anti-mouse or anti-rabbit IgG (1:1000). The mouse monoclonal antibodies gave no background signal in the non-transformants because of nonspecific antibody binding under the conditions described above. For the staining of endosomal and lysosomal compartments, amoebae were pulsed with either 2 mg/ml FITC-dextran (Sigma) for 10 min or LysoTracker Red DND-99 (Molecular Probes) (1:500) for 12 h at 35 °C. Samples were examined on a...

**1** The abbreviations used are: CP, cysteine proteinase; FITC, fluorescein isothiocyanate; PPV, prephagosomal vacuole; GFP, green fluorescent protein; PBS, phosphate-buffered saline; HA, hemagglutinin; EhRab5, *Entamoeba histolytica* Rab5; EhRab7A, *Entamoeba histolytica* Rab7A.
Zeiss LSM510 confocal laser-scanning microscope. Images were further analyzed using LSM510 software.

**Time-lapse Microscopy**—Amoeba transformants expressing GFP-EhRab5 and GFP-EhRab7A were plated onto a 35-mm glass-bottom culture dish (D111100, Matsunami Glass Ind. Inc., Osaka, Japan) to settle amoebae at 30 °C. After the medium was removed, the glass chamber was enclosed by a glass coverslip. Time-lapse microscopy was performed with a Leica AS MDW system on a Leica DM IRE2 inverted microscope. Images of 18 slices (1.5 μm apart on the z-axis) were captured at 2.85-s intervals. This z-spacing was optimized to: 1) monitor the entire depth of amoebae from the top to the bottom, and 2) to accomplish fast capturing of a moving amoeba. Obtained raw images were further deconvoluted using Leica Deblur software. For each time point, images were three-dimensionally reconstituted and only a selected plane containing a PPV or a GFP-EhRab5-associated compartment was shown.

**Immunoelectron Microscopy**—Immunoelectron microscopy was performed by pre-embedding labeling method (44). Amoebae were transferred to slide glass and incubated with red blood cells for 10 min as described above. Samples were prefixed with 3.7% paraformaldehyde, PBS for 20 min, and then incubated with 0.1 M glycine, PBS, and permeabilized with 0.1% Triton X-100. Samples were reacted with anti-amoebapore A (1:50), and subsequently with a goat anti-rabbit IgG conjugated with 5-nm gold (1:30). These cells were embedded into 2% soft agar, and further fixed with 0.1% OsO4, PBS for 30 min followed by dehydration, and embedded in Epon 812 (TAAB Laboratories Equipment, LTD., UK). Ultrathin sections were made on an LKB-ultratome (LKB-Produkter, Bromma, Sweden), and sections were stained with uranyl acetate and examined with a Hitachi-H-700 electron microscope.

**Measurement of FITC-dextran Uptake**—Transformants were cultured in BI-S-33 medium containing 2 mg/ml of FITC-dextran for given periods at 35 °C. After the incubation, cells were washed three times
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RESULTS

Identification of Entamoeba Homologues of Rab5 and Rab7 (EhRab5 and EhRab7A)—We isolated cDNAs coding for a putative homologue of Rab5 and Rab7, and designated them EhRab5 and EhRab7A, respectively. EhRab5 and EhRab7A showed 45 and 48% identity to mammalian Rab5 and Rab7, respectively. The effector region and a2 helix loop, which are important to the specificity of Rab proteins (45), were well conserved among mammalians, yeasts, and *E. histolytica* (Fig. 2).

To examine whether the amoebic Rab5 and Rab7A play a role similar to that in other organisms, we attempted to rescue defects of a yeast Δypt5Δvps21 mutant (46, 47) and Δypt7 mutant (14) through ectopic expression of EhRab5 and EhRab7A, respectively. Overexpression of EhRab5 on a single-copy plasmid under the regulation of a GAL1 promoter did not complement either the fragmented vacuole morphology or a temperature-sensitive growth defect in Δypt5Δvps21 cells (data not shown). Neither did overexpression of EhRab7A in the Δypt7 mutant rescue vacuole fragmentation (data not shown). These results indicate that amoebic Rab5 and Rab7A play a role distinct from that of yeast Ypt51p and Ypt7p.

Dynamics of EhRab5 and EhRab7A during Phagocytosis and Identification of Unique PPV Associated with EhRab5 and EhRab7A—We examined the subcellular localization of EhRab5 and EhRab7A during phagocytosis of red blood cells. We constructed a stable transformant that constitutively expressed an 3HA-tagged EhRab5 and a 3Myc-tagged EhRab7A. EhRab5 and EhRab7A were estimated to be overexpressed by 3–5- and 1.5–2-fold, respectively, in the transformant when compared with wild-type cells by quantitation of immunoblots using an antibody raised against recombinant EhRab5 and EhRab7A (data not shown). Neither expression of the epitope-tagged EhRab5 alone nor co-expression of both epitope-tagged EhRab5 and EhRab7A affected cell growth or morphology (see below and Fig. 8A).

Immunofluorescence imaging using anti-EhRab5 and anti-Myc antibody, the latter of which reacts with 3Myc-tagged EhRab7A, showed that, at steady state (i.e. without red blood cells), EhRab5 and EhRab7A were localized to small non-overlapping vesicles throughout the cytoplasm (Fig. 3, A–D). The distribution of EhRab5 and EhRab7A dramatically changed upon incubation with red blood cells. After 5 min, large vacuoles (4.0 ± 0.9 µm in diameter) that colocalized with both EhRab5 and EhRab7A emerged (Fig. 3, E–H). At 10 min, EhRab5 began to dissociate from some of these vacuoles, whereas EhRab7A remained associated with them (Fig. 3, I–L). These EhRab5/EhRab7A-positive vacuoles also formed in the amoebae that did not ingest red blood cells (a trophozoite in Fig. 3, E–H, and a trophozoite on the right in Fig. 3, I–L). We designated these vacuoles PPV as this compartment emerged prior to fusion with phagosomes (see below). At 30 min, when the amoebae ingested an average of 3–4 red blood cells per cell, EhRab5/EhRab7A double-positive PPV disappeared and EhRab5 dispersed into the cytosol as seen at steady state. Approximately 40% of phagocytosed red blood cells were surrounded by EhRab7A (Fig. 3, N, P, and Q). EhRab5 was not localized to phagosomes containing red blood cells at any time point (Fig. 3, A, E, I, and M), which is in good contrast to the dynamics shown for mammalian Rab5 in macrophages, where phagosomes are simultaneously associated with both Rab5 and Rab7 (4).

To unequivocally demonstrate the dynamics of the maturation of PPV and phagosomes, we counted (i) EhRab5/EhRab7A double-positive PPV, (ii) EhRab7A single-positive PPV, (iii) EhRab7A positive phagosomes, and (iv) EhRab7A negative phagosomes (Fig. 3Q). The number of these vacuoles per cell changed during the course of phagocytosis. The number of EhRab5/EhRab7A double-positive PPV peaked at 5 min and gradually decreased after 10 min, whereas the number of EhRab7A single-positive PPV increased at 5–10 min, and remained elevated up to 30 min. The proportion of EhRab5/EhRab7A double-positive PPV among all PPV (i.e. i(i + ii)) sharply decreased between 5 and 30 min (78, 37, and 8% at 5, 10, and 30 min, respectively). The number of phagosomes increased linearly during 30 min (0.8 per cell at 5 min to 5.3 per cell at 30 min). However, the proportion of EhRab7A-positive phagosomes among all phagosomes (i.e. iii/(iii + iv)) did not significantly change during the course (30–40%). These results support the following model: 1) upon interaction with red blood cells, EhRab5/EhRab7A double-positive PPV forms; 2) EhRab5 is dissociated from EhRab5/EhRab7A double-positive PPV; 3)
Fig. 3. Subcellular localization of \(Eh\)Rab5 and \(Eh\)Rab7A changed during red blood cell phagocytosis. A–P, subcellular localization of \(Eh\)Rab5 and \(Eh\)Rab7A was examined by immunofluorescence assay using the amoeba transformant co-expressing 3HA-tagged \(Eh\)Rab5 and 3Myc-tagged \(Eh\)Rab7A in the absence of red blood cells (A–D), or after 5 (E–H), 10 (I–L), and 30 min (M–P) incubation with red blood cells. Localization of \(Eh\)Rab5 and \(Eh\)Rab7A was examined with anti-\(Eh\)Rab5 antibody (green; A, E, I, and M) and anti-Myc monoclonal antibody (red; B, F, J, and N), respectively. Merged images of \(Eh\)Rab5 and \(Eh\)Rab7A (C, G, K, and O) and phase-contrast images under transmission light (D, H, L, and P) are also shown. Large arrowheads show \(Eh\)Rab5/\(Eh\)Rab7A-containing PPV (E–K). Small arrowheads (N–P) show \(Eh\)Rab7A-positive phagosomes. Thick arrows (J, K, N, O, and P) indicate \(Eh\)Rab7A-PPV, not associated with \(Eh\)Rab5. A thin arrow (L) indicates an engulfed red blood cell associated with neither \(Eh\)Rab5 nor \(Eh\)Rab7A. Q, quantitative analysis of \(Eh\)Rab5 and \(Eh\)Rab7A localization to PPV and phagosomes during erythrophagocytosis. The number of \(Eh\)Rab5/\(Eh\)Rab7A double-positive PPV (open bars; also marked as 5/7A-PPV), \(Eh\)Rab7A single-positive PPV (gray bars; 7A-PPV), \(Eh\)Rab7A-positive phagosomes (hatched bars; 7A-phagosome), and \(Eh\)Rab7A-negative phagosomes (filled bars; phagosome) per cell is shown at 5, 10, and 30 min after the addition of red blood cells. R–U, subcellular localization of \(Eh\)Rab7A was examined by immunofluorescence assay using wild-type amoebae in the absence of red blood cells (R and S) or after a 10-min (T and U) incubation with red blood cells. Panels S and U show phase images of panels R and T, respectively. Arrowheads in T and U depict PPV. V and W, three-dimensional sections of the amoeba containing red blood cell showing the presence of red blood cells in phagosomes, but not in PPV. Localization of PPV and red blood cells was examined with anti-\(Eh\)Rab5 antibody (green, arrowheads) and anti-band 3 antibody (arrows, red), respectively. Among 17 z-sections (1-µm intervals) obtained with confocal laser scanning microscopy, only one representative xy section, together with selected yz (green line), and yz (red line) sections, are shown. W shows a phase image of V.

\(Eh\)Rab7A is subsequently targeted to phagosomes; and 4) \(Eh\)Rab7A is finally dissociated from phagosomes.

We also verified that PPV was not an artifically misidentified phagosome, i.e. the phagosome that contains debris of red blood cells, but is not stained by dianminobenzidine because of loss of its content. To exclude this possibility, we used an antibody raised against a major component of the membrane cytoskeleton of red blood cells, band 3 (48). The anti-band 3 antibody clearly reacted with red blood cells in phagosomes (Fig. 3V, red, arrow), whereas none of the \(Eh\)Rab5-positive PPV was reacted with the antibody (green, arrowheads). In addition, localization of red blood cells by dianminobenzidine staining or anti-band 3 antibody agreed very well (Fig. 3, V and W). These results clearly showed that PPV are distinct from phagosomes. The PPV formation was not a secondary defect caused by expression of epitope-tagged \(Eh\)Rab5 and \(Eh\)Rab7A because it was also observed in wild-type amoebae at a comparable frequency, as detected by the antibodies raised against recombi-
EhRab5 is not associated with endosomes or lysosomes but exhibits cross-talk with these compartments during maturation of PPV. To see whether EhRab5 is associated with endosomes, we examined colocalization of an endocytosed fluid-phase marker, FITC-dextran, and EhRab5. Amoebae were either incubated with FITC-dextran for 10 min to label the early endosomes or incubated with FITC-dextran for 10 min and further chased without FITC-dextran for 45 min to label the late endosomes, and then subjected to immunofluorescence assay using anti-HA antibody that recognizes EhRab5 (Fig. 4A). Endocytosed FITC-dextran and EhRab5 did not colocalize at either 10-min pulse (Fig. 4A) or at the 10-min pulse followed by a 45-min chase (data not shown). These findings imply that the EhRab5-positive compartment is neither early nor late endosomes.

When amoebae were simultaneously incubated with red blood cells and FITC-dextran for 10 min, 30% of PPV contained endocytosed FITC-dextran (Fig. 4B). When the amoebae were pulsed with FITC-dextran for 10 min and chased for 45 min to label the late endosomes, and further incubated with red blood cells for 10 min, the extent of colocalization of FITC-dextran and PPV was comparable (26%; data not shown). These results suggest that PPV fuse with both early and late endosomes during maturation.

To assess where and how PPV are formed during phagocytosis, we examined the dynamics of EhRab5 using the amoeba transformant expressing GFP-EhRab5 under time-lapse microscopy. Images of 18 planes of the z-section with 1.5-μm intervals to cover from the top to the bottom of the cell were recorded at 2.85-s intervals. This allowed us to evaluate the detailed dynamism of PPV formation. After a few minutes of coincubation with red blood cells, an EhRab5-positive vacuole suddenly emerged in less than 20 s. Neither plasma membrane invagination nor ruffling were observed during this period, suggesting that PPV forms de novo (Fig. 5).

We also excluded a possibility that PPVs are micropinosomes or phagosomes. First, the fact that only a minor proportion of PPV contained FITC-dextran at 10 min (Fig. 4B) suggests that PPVs are not formed by invagination of the plasma membrane-like macropinosomes, which form by the closure of membrane ruffles and contain a fluid-phase marker (49, 50). Second, PPV is formed in a range of 10 s (Fig. 5), much faster than macropinosomes or phagosomes (49, 51). Membrane closure of macropinosomes and phagosomes was previously shown to occur in 1 and 5 min, respectively. Third, the major Gal/GalNAc lectin on the plasma membrane was abundantly demonstrated in phagosomes by proteomic analysis of phagosome proteins during the course of phagosome maturation (from 0 min to 2 h after ingestion) but was not demonstrated on PPV by immunofluorescence study using a specific monoclonal antibody against heavy or intermediate lectin subunits (data not shown). These results strongly argue against two possibilities: 1) PPV originates from the plasma membrane, and 2) PPV is a remnant of phagosomes.

Acidification of phagosomes has been shown to occur by fusion with late endosomes and lysosomes in mammalian cells (52). We examined by using LysoTracker Red, a membrane-permeable dye accumulated in acidic organelles (53), whether the PPV and phagosomes of the amoeba are acidified during maturation. Amoebae were pulsed with LysoTracker and then subjected to immunofluorescence assay. At steady state, neither EhRab5 (Fig. 6A, left) nor EhRab7A (Fig. 6A, right), probed with anti-HA or anti-Myc antibody, respectively, colocalized with LysoTracker. After a 5–10-min incubation with red blood cells when EhRab7A-positive PPV were formed, only 20–30% of PPV contained LysoTracker, suggesting that PPV were only partially acidified in the early stage (Fig. 6, B, upper panels, and C, data at 5 min not shown). After 30–40 min, a large proportion (50–70%) of PPV became acidified (Fig. 6, B, lower panels, and C).

PPVs are involved in the transport of amoebapore to phagosomes. We then examined which cargo proteins were transported via PPV. Among several hydrolases and membrane-permeabilizing factors involved in the degradation of internalized host cells and microorganisms, e.g. CP (26), amoebapore (27), lysozyme (54), and phospholipases (55), we tested whether amoebapore A and CP2 were transported to phagosomes via PPV. Immunostaining of amoebapore and CP2 using specific antisera showed similar patterns to those obtained with LysoTracker in the absence of red blood cells (Fig. 7A, 0

**FIG. 4.** Immunofluorescent micrographs showing cross-talk between early endosomes and PPV. A, the amoebae were pulsed with 2 mg/ml FITC-dextran (green) for 10 min, washed with PBS, and then subjected to immunofluorescence assay using anti-HA antibody to probe 3HA-tagged EhRab5 (red). Yellow arrowheads indicate endocytosed FITC-dextran. B, the amoebae were pulsed with FITC-dextran in the presence of red blood cells for 10 min, and then subjected to immunofluorescence assay. A yellow or white arrow indicates an EhRab5-associated PPV that contains or does not contain endocytosed FITC-dextran, respectively. A yellow arrowhead depicts the endocytosed FITC-dextran that is not associated with EhRab5 in the cytoplasm. Bars, 10 μm.

**FIG. 5.** Time-lapse micrographs of an amoeba expressing GFP-EhRab5, showing de novo formation of PPV. Amoebae were mixed with red blood cells, and then images of a stack of 18 sections along the z-axis (every 1.5 μm) were immediately recorded every 2.85 s. From each time point, a representative section showing EhRab5-associated vesicle or vacuole during the course of PPV formation was chosen to show the de novo generation of PPV at a site indicated by the arrowheads. Times in seconds are also shown. Bars, 10 μm.
Expression of EhRab5 Wild Type or Mutants Influences Cell Growth, Ingestion of Red Blood Cells, and Amoebapore Transport to Phagosomes but Not Endocytosis—To further examine the specific role of EhRab5 and PPV, we introduced a constitutively active GTP form (EhRab5Q67L) or an inactive GDP form (EhRab5S22N) mutant of EhRab5 into wild-type amoeba. Introduction of neither wild-type EhRab5 nor EhRab5S22N affected the amoeba growth compared with the vector control independent of coexpression of EhRab7A (Fig. 8A). In contrast, expression of EhRab5Q67L unexpectedly caused a severe growth defect. This is the first case of a growth defect caused by the expression of a mutant Rab5.

We also studied the effects of expression of wild-type and mutant EhRab5 on phagocytosis of red blood cells. The number of red blood cells engulfed by the amoebae at 10, 20, or 30 min was counted (Fig. 8B). Expression of wild-type EhRab5 accelerated engulfment of red blood cells by 1.4–2.2-fold, whereas expression of either the EhRab5Q67L or EhRab5S22N mutant inhibited the efficiency of phagocytosis by 50–70% compared with the control transformant.

Next, we assessed whether expression of EhRab5 wild-type or mutants influences the transport of cargo proteins, e.g. amoebapore, to phagosomes. Efficiency of the amoebapore transport was evaluated by calculating percentages of phagocytosed red blood cells that colocalized with amoebapore (Fig. 8C). In the control transformant cells, 67.0 ± 7.5% of engulfed red blood cells colocalized with amoebapore at 30 min of incubation, whereas 87 ± 2.3% of the ingested red blood cells colocalized with amoebapore in wild-type EhRab5-expressing cells (p < 0.01). In contrast, the expression of EhRab5Q67L reduced efficiency of the amoebapore transport to 45 ± 3.0% (p < 0.05), whereas no significant change was observed in the EhRab5S22N-expressing transformant (58 ± 2.8%, p > 0.1). These data indicate that overexpression of wild-type EhRab5 or the EhRab5Q67L mutant increased or interfered with the amoebapore transport to phagosomes, respectively. Neither fluid-phase nor receptor-mediated endocytosis (56), as indi-

Fig. 6. Acidification of EhRab5/EhRab7A-associated PPV and phagosomes. A, localization of EhRab5 (green) and LysoTracker (red) (left panel), and EhRab7A (green) and LysoTracker (red) (right panel) in the absence of red blood cells. Amoebae were pulsed with LysoTracker overnight and subjected to immunofluorescence assay with anti-HA (for the absence of red blood cells. Amoebae were pulsed with LysoTracker, but not associated with blood cell incubation. Yellow arrows indicate phagosomes that contain LysoTracker, but not associated with EhRab7A-positive PPV containing LysoTracker. White arrowheads (30 min) indicate EhRab7A-positive PPV containing LysoTracker. White arrowheads indicate phagosomes containing LysoTracker and are also associated with EhRab7A. Yellow arrows indicate phagosomes containing LysoTracker, but not associated with EhRab7A. Bars, 10 μm. C, quantitative analysis of PPV acidification. The number of LysoTracker-associated (filled bars) or non-associated PPV (open bars) per amoeba is shown together with the percentages of the acidified PPV (circles and a line). Error bars represent S.D. of three independent experiments.

min), suggesting that both amoebapore and CP2 were contained in the lysosomes at steady state. The subcellular localization of both amoebapore and LysoTracker changed during erythrophagocytosis. At 10 min, 80% of acidified EhRab7A-positive PPV were associated with amoebapore (Fig. 7, A, 10 min, and C). At 30 min, all acidified EhRab7A-positive PPV remained amoebapore-positive (Fig. 7C, 30 min). In contrast, amoebapore and LysoTracker did not perfectly overlap on phagosomes at 30 min; all combinations of amoebapore and LysoTracker positive or negative phagosomes were seen (Fig. 7A, 30 min). As the number of total phagosomes increased during incubation with red blood cells, the number of amoebapore- or LysoTracker-positive phagosomes increased in parallel (Fig. 7D). However, the number of EhRab7A-associated phagosomes did not increase after 30 min; the percentage of EhRab7A-positive phagosomes transiently increased at 20 min and then decreased (i.e. 20, 31, 25, and 19% at 10, 20, 30, and 50 min), consistent with the notion that EhRab7A was dissociated from phagosomes at this stage. The kinetics of CP2 was indistinguishable from that of amoebapore (data not shown). These results indicate that amoebapore and CP2 were transported from lysosomes to phagosomes via PPV.

We noticed that amoebapore was concentrated in the peripheral part of PPV, and not evenly distributed in the vacuole (e.g. Fig. 7A, 10 min). An immunoelectron micrograph using an anti-amoebapore A antibody further documented detailed localization of amoebapore in the PPV (Fig. 7B). At the 10-min addition of red blood cells, gold particles were detected on an amorphous structure that partially occupies the lumen. Furthermore, the amoebapore-containing vacuole included membrane structures (Fig. 7B, arrow). The concentrated localization of amoebapore within PPV was similar to that observed for endocytosed FITC-dextran (Fig. 4B).

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cated by FITC-dextran (Fig. 8D) or lactoferrin (data not shown) internalization, respectively, was influenced by expression of wild-type or mutant EhRab5 up to 3 h. Together with the lack of colocalization of EhRab5 and FITC-dextran shown above (Fig. 4A), these results also support the premise that EhRab5 is unlikely involved in endocytosis. In addition, the efflux of the internalized fluid-phase marker was not affected in EhRab5 mutants (data not shown).

EhRab5 Plays an Essential Role in the Formation of PPV—Subcellular localization of EhRab5 mutants was examined to assess possible reasons for a defect in growth, phagocytosis, and amoebapore transport to phagosomes. Confocal immunofluorescence micrographs showed that both EhRab5Q67L and EhRab5S22N, probed with anti-EhRab5 antibody, were localized to small vesicular-like structures that resemble those observed for wild-type EhRab5 and EhRab7A at steady state (data not shown). After a 10-min incubation with red blood cells, in contrast to wild-type EhRab5, EhRab5Q67L- or EhRab5S22N-associated vacuoles were not observed; their localization appeared to be identical to that at steady state (Fig.
These results, together with the data shown above, suggest that EhRab5 is essential for the formation of PPV, which is required for the efficient phagocytosis and transport of the degradative proteins to phagosomes.

**DISCUSSION**

Although EhRab5 showed about 50% identity to the mammalian and yeast counterparts and the putative effector domain is very similar between *E. histolytica* and other organisms, the function of the amoebic Rab5 appears to be divergent from that of the mammalian and yeast homologues. First, whereas the mammalian and yeast Rab5/Ypt51p play a role in endocytosis, *Eh* Rab5 is involved exclusively in phagocytosis, but not in endocytosis. This has been shown by the absence of colocalization of EhRab5 and the endocytosed FITC-dextran (Fig. 4), and also by a lack of augmented uptake of the endocytosis marker by expression of wild-type or the dominant active EhRab5 mutant (Fig. 8D). Second, the localization of EhRab5 and its association to 3–5-μm translucent PPV, which has not been described in other organisms, is unique to this organism. In the mammalian cells, Rab5 is localized to the early endosomes, and early endosomes directly fuse with primary phagosomes during phagocytosis (4, 7), whereas *Eh* Rab5 is not localized to phagosomes at any stages of phagocytosis (Fig. 3). Instead, *Eh* Rab5 is localized, in conjunction with *Eh* Rab7A, to PPV before these vacuoles fuse with phagosomes containing red blood cells. Third, in contrast to mammals, where similar phenotypes were observed in transformants expressing wild-type and GDP-mutant Rab5 and opposite phenotypes were observed in the GDP-mutant Rab5 expressing transformants (5), expression of *Eh* Rab5 GTP or GDP mutant showed a similar defect in erythrophagocytosis and PPV formation (Fig. 8). This may indicate that requirement of GTP hydrolysis by Rab5 for membrane fusion may differ between the amoeba and other organisms. Fourth,
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**EhRab5 does not functionally complement the yeast Δypt51 mutant.** This is in good contrast to the yeast and mammalian counterparts, which are virtually interchangeable; Ypt51p expressed in a mammalian cell was properly localized to endosomes and accelerated endocytosis (6). Fifth, compared with other organisms including mammals, plants, yeasts, and a parasitic protist *Trypanosoma brucei*, which have been shown to possess 2–5 Rab5 isotypes with distinct tissue and organelle distribution or developmental stage-specific expression (46, 57–59), *E. histolytica* possesses only a single Rab5 gene based on our thorough search of the genome data base (data not shown). Altogether, *EhRab5* represents a unique Rab5 showing diverse localization and functions.

We have identified and characterized an unprecedented vacuole "PPV," which is co-associated with *EhRab5* and *EhRab7A* at the early stage of its formation and becomes dissociated by *EhRab5* during maturation. We have shown that PPV were formed *de novo* in a very short time, and then acidified, in a time-dependent fashion during phagocytosis, by the fusion of lysosomes, which contain at least two independent degradative proteins, i.e. CP2 and amoebapore A (Figs. 5–7). We propose that PPV serves as a compartment for the temporal storage, processing, and/or activation of hydrolytic enzymes and lytic peptides before fusion with phagosomes containing ingested host cells and microorganisms. In mammalian cells, a newly formed phagosome is subjected to gradual maturation by continuous exchange of its contents via sequential fusion with the early and late endosomal compartments, leading to the formation of acidified phagolysosome (60). In contrast, in the amoeba, PPV apparently serves as a reservoir of digestive enzymes (Fig. 7) and endosomal content (Fig. 4) prior to fusion with phagosomes (Fig. 7). An immunoelectron micrograph showed that the vacuole containing amoebapore were enclosed by another membrane structure (Fig. 7B). Although multivesicular vacuoles have been previously reported in *Entamoeba* (61), this is the first demonstration of a particular protein within these multivesicular vacuoles. In mammalian and yeast cells, multivesicular bodies have been regarded as late endosomes, in which proteins to be transported to lysosomal lumen are selectively packed into internal vesicles (62). These observations imply that some proteins targeted to phagosomes are selectively included in PPV. We have recently identified the homologue of retromer, which functions in retrieval of receptor proteins from late endosomes to the trans-Golgi network (63). The observation that one of retromer components, *EhVps26*, is localized on PPV might imply that PPV had a similar function to late endosomes/multivesicular bodies. As far as we are aware, such a "preparatory" organelle has not previously been described and may represent a novel cellular compartment.

The formation of PPV was induced most efficiently by interaction with red blood cells. A membrane ghost, but not a soluble fraction, of red blood cells also induced the formation (data not shown). However, latex beads, yeasts, and *Escherichia coli* cells induced the formation of PPV to a much lesser extent (data not shown). Thus, Rab5 recruitment to PPV in this parasite may occur predominantly in a case of the engulfment of red blood cells. PPV were also observed in cells that did not initiate engulfment of red blood cells (Fig. 3, E–L). These findings suggest that interaction with red blood cells, but not engulfment *per se*, is sufficient for the induction of PPV formation.

One intriguing hypothesis to explain why PPV formation is specifically induced by red blood cells is that PPV is required for the degradation and/or detoxification of the content of red blood cells. It was previously reported that amoebae recognizes surface glycans with Galβ1–4GlcNAc terminal glycosphingolipid on red blood cells (64) by a Gal/GalNAc-inhibitable lectin (21, 22). The Galβ1–4GlcNAc terminal glycosphingolipid is absent on the surface of latex beads, yeast, and *E. coli* (65, 66). It has also been demonstrated in macrophages that the phagocytosis-induced response also depends on receptors (67). For example, phagocytosis via the Fc receptor lead to the production of proinflammatory molecules such as reactive oxygen intermediates, whereas phagocytosis involving mannose receptor produced proinflammatory cytokines including interleukin-1β and tumor necrosis factor-α (68). In contrast, phagocytosis via the complement receptor did not elicit release of inflammatory mediators (69). Actin and microtubules were shown to be important for the complement receptor system, whereas two regulatory proteins of actin cytoskeleton, vinculin and proxilin, were not necessary for the mannose receptor system, indicating diversity of the receptor-response relationship during phagocytosis (70).

In view of the signals necessary for PPV formation, we also noted that a phosphatidylinositol 3-kinase inhibitor, wortmannin at 100 nm, abolished both ingestion, as previously reported (71), and PPV formation (data not shown). This finding also supports a tight correlation between ingestion of red blood cells and formation of PPV. The fact that expression of wild-type or GTP mutant *EhRab5* resulted in augmented or diminished ingestion of red blood cells, respectively, also supports the premise that signal transduces from PPV to an initial site of engulfment. However, whether a phagosome-associated phosphatidylinositol 3-kinase is present in the amoeba, as shown in mammals (Vps34) (12) and what effector proteins (*e.g.* EEA1 in mammals) (72, 73) are recruited to the phagosomes of the amoeba in a phosphatidylinositol 3-kinase-dependent manner remain unknown.

We have shown detailed quantitative data on how the maturation of PPV and phagosomes occur during erythropagocytosis (Figs. 3Q, 6C, and 7, C and D). In contrast to the gradual and continuous acidification of PPV, which occurs in parallel with recruitment of digestive enzymes to PPV, acidification of phagosomes appears to be interrupted or, more likely, reversed by neutralization, which synchronizes with the dissociation of *EhRab7A* from phagosomes (Fig. 7D). A few lines of evidence suggest that neutralization of phagosomes takes place soon after the content of PPV is transported to phagosomes. First, the percentage of acidified phagosomes remained unchanged between 20 and 50 min after ingestion (*e.g.* 43 and 38%, at 20 and 50 min, respectively). Second, the percentage of acidified phagosomes was significantly lower than that of amoebapore-containing phagosomes at all time points (*e.g.* 58 and 71% at 20 and 50 min).

We propose here a model by which *EhRab5* and *EhRab7A* coordinately regulate membrane fusion during phagocytosis. Upon the interaction of red blood cells with the amoeba plasma membrane, independent *EhRab5* or *EhRab7A*-associated vesicles receive a signal, in a phosphatidylinositol 3-phosphate-dependent manner, presumably from the Gal/GalNAc-specific surface lectin or a not yet identified plasma membrane receptor, which initiates subsequent sorting and reorganization of these compartments. *EhRab5* vesicles start to heterotypically fuse with *EhRab7A*-associated vesicles, and then form PPV. PPV simultaneously fuse with lysosomes containing amoebapore and hydrolases. *EhRab5* is then dissociated from PPV before the content of *EhRab7A*-associated PPV is targeted to phagosomes. Because the size of phagosomes did not increase after *EhRab7A* was transported from PPV to phagosomes, the direct fusion between PPV and phagosome likely does not oc-

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cur. We propose that transfer of the content of PPV involves vesicular trafficking, i.e., budding from PPV followed by fusion of these vesicles to phagosomes. Once the content of PPV is transferred to phagosomes, EhRab7A is dissociated from phagosomes, whereas digestive proteins remain in phagosomes. Neutralization of phagosomes also plays a major role in PPV dissociation. After degradation of internalized materials, membrane recycling from phagosomes also occurs via the budding of recycling vesicles (74). Finally, the molecular dissection of a unique function of EhRab5 and a novel EhRab5-associated compartment in this parasite may shed light on the Entamoeba-specific phagocytic mechanisms closely related to its virulence competence.

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