Endoplasmic reticulum-resident Rab8A GTPase is involved in phagocytosis in the protozoan parasite Entamoeba histolytica

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Introduction

Phagocytosis is a ubiquitous mechanism that is evolutionarily conserved in a wide range of eukaryotes. It comprises several key steps: receptor-mediated recognition of the particle (prey), signal transduction, rearrangement of the cytoskeleton and remodelling of the membrane and contents (Vieira et al., 2002; Stuart and Ezekowitz, 2005). The surface receptors for a variety of targets activate a number of signalling cascades leading to target-dependent responses (Kinchen and Ezekowitz, 2005).

Entamoeba histolytica is a protozoan parasite and the causative agent of amoebiasis in humans. Phagocytosis plays an important role in its pathogenesis as it participates in the killing of and evasion from host immune cells (Ralston and Petri, 2011; Faust and Guillen, 2012). Two types of ingestion of cells and particles by E. histolytica are proposed. Phagocytosis is a process by which dead cells and large particles such as bacteria are internalized as a whole. Induction of apoptosis to host cells by a contact of E. histolytica has been reported previously (Huston et al., 2003; Faust et al., 2011). The Gal/GaINAc lectin and cysteine protease A5 are required for phagocytosis (Mann et al., 1991). Trophozoites induce apoptosis to host cells by contact (Huston et al., 2003; Faust et al., 2011), recognize phosphatidylinerse exposed on the apoptotic and necrotic host cells (Boettner et al., 2005) and internalize them by phagocytosis. In contrast, trogocytosis, recently described by Ralston et al. (2014), is a process in which a cell nibbles pieces of a living host cell, eventually leading to death by the loss of membrane integrity and mitochondrial potential (Guillen, 2014; Ralston et al., 2014; Ralston, 2015). This process requires Gal/GaINAc lectin, actin rearrangement and PI3K signaling of trophozoites by the loss of membrane integrity and...
Amoebic Rab8 in phagocytosis

Results

Down-regulation of phagocytosis by repression of Rab8 expression by gene silencing

Our previous proteomic study of isolated phagosomes has revealed that 14 amoebic Rab proteins are localized to phagosomes (Okada et al., 2005; 2006). In order to screen Rab proteins that are involved in the engulfment and phagosome maturation per se, E. histolytica strains in which expression of each EhRab protein of the all phagosome-associated EhRabs except for EhRab7E, which is not expressed in G3 strain, along with two additional Rab7 isoforms, EhRab7G and 7H, was silenced by antisense small RNA-mediated transcriptional gene silencing were created (Bracha et al., 2006; Mi-ichi et al., 2011; Zhang et al., 2011). Among 15 EhRab proteins, the specific repression of EhRab7B (EHI_081330), EhRab7D (EHI_082070), EhRab7G (EHI_187090), EhRab7H (EHI_005900), EhRab8A (EHI_199820), EhRab11B (EHI_107250) and EhRab11C (EHI_161030) was achieved. However, gene silencing of EhRab1A (EHI_108610), EhRab5 (EHI_026420), EhRab7A (EHI_192810), EhRab7C (EHI_189990), EhRabC1 (EHI_153690), EhRabC2 (EHI_045550), EhRabC3 (EHI_143650) and EhRabX17 (EHI_042250) was not successful despite repeated trials, presumably because of their essentiality (data not shown). In this study, we focused on the analysis of EhRab8A. Analyses of other EhRab gene-silenced strains will be reported elsewhere.

The specific repression of EhRab8A expression was confirmed by real-time polymerase chain reaction (PCR) of the corresponding cDNA (Fig. 1A). The expression level of EhRab8A mRNA was decreased 35-fold ($P<0.015$) in EhRab8A gene-silenced strain compared with that of the mock control. Moreover, expression of the paralogous Rab8 isotype, EhRab8B (EHI_127380), which has a 54% nucleotide identity to EhRab8A, was not affected by EhRab8A gene silencing ($P>0.05$). Loss of EhRab8A protein was further confirmed by immunoblot analysis with a polyclonal antibody raised against recombinant EhRab8A protein (Fig. 1B). EhRab8A gene-silenced strain showed growth retardation compared with mock control (the doubling time: $34\pm6.0$ h for EhRab8A gene-silenced strain; $26\pm11$ h for mock control, $P<0.05$) (Fig. 1C).

EhRab8A gene-silenced strain showed a remarkable defect in phagocytosis of various prey, that is, carboxylated latex beads, erythrocytes and Escherichia coli cells (Fig. 1D). Flow cytometric analysis revealed that EhRab8A gene-silenced trophozoites ingested carboxylated latex beads, erythrocytes and E. coli cells less efficiently by 46% ± 10% (at 30 min, $P<0.05$ compared with the mock control cells, $n=3$), 26% ± 11% (at 20 min, $P<0.01$) and 83% ± 6% (at 30 min, $P<0.01$) respectively.
Thus, EhRab8A is involved in phagocytosis of a broad range of targets.

Reduced phagocytosis in EhRab8A gene-silenced strain because of decreased adherence

Using a rosette formation assay described in the Experimental Procedures section, we evaluated whether the decreased phagocytosis in the EhRab8A gene-silenced cells was because of reduced adherence to the target (Fig. 2A). We quantified the number of trophozoites adherent to five or more erythrocytes after a 30 min incubation on ice. Under that condition, where the amoebae did not ingest erythrocytes (Huston et al., 2003), 30% ± 8% of the EhRab8A gene-silenced cells adhered to ≥5 erythrocytes,
whereas 47% ± 3% of the mock control cells attached to ≥ 5 erythrocytes (n = 100 for each, P = 0.026) (Fig. 2B). These data suggested that the EhRab8A gene-silenced transformant was less capable of adherence to erythrocytes.

It has been shown that amoebic host recognition and attachment are mediated by a range of PM-associated proteins, including Gal/GalNAc lectin (Petri et al., 1989; Cheng et al., 1998), TMK96 (Boettner et al., 2008), STIRP (MacFarlane et al., 2007), KERP1 (Santi-Rocca et al., 2008) and SREHP (Stanley et al., 1990). To examine the surface protein profiles of EhRab8A gene-silenced and mock control cells, the PMs of live trophozoites were labelled with biotin and visualized on immunoblots using streptavidin–horseradish peroxidase (HRP) after SDS-PAGE (Zhao et al., 2004) (Fig. 2C). The intensities of at least three major bands, corresponding to 200, 70 and 30 kDa (arrowheads) were reduced in the EhRab8A gs strain.
was conducted. However, no difference was observed in the staining of the PM of the heavy (170 kDa) and intermediate (150 kDa) subunits of Gal/GalNAc lectin by the corresponding specific antibodies (Petri et al., 1990; Cheng et al., 2001) (Fig. S1A and B). Surface staining with anti-KERP1 (21 kDa) antibody also showed no difference between mock control and EhRab8A gene-silenced cells (Seigneur et al., 2005) (Fig. S1C). Furthermore, the apparent molecular mass of the missing bands in the EhRab8A gene-silenced strain did not match the sizes of the aforementioned proteins (TMK96, 146 kDa; STIRP, 280 kDa; SREHP, 50 kDa). No difference was observed in the profiles of the whole cell lysate stained with SYPRO Ruby after SDS-PAGE between the EhRab8A gene-silenced strain and mock control cells (Fig. 2C).

**EhRab8A localized to the ER, but not to the trans-Golgi network nor to lysosomes**

EhRab8A is homologous to yeast Sec4p and human Rab8, with 49–53% amino acid identity (Juarez et al., 2001; Saito-Nakano et al., 2005) (Fig. S2). Yeast Sec4p and human Rab8 are known to be involved in trafficking from the trans-Golgi network (TGN) to the PM (Salminen and Novick, 1987; Huber et al., 1993). Further, colocalization of Rab8 with Rab11, which is the upstream regulator of Rab8, has also been reported (Feng et al., 2012; Wang et al., 2012). Thus, we examined whether EhRab8A was colocalized with EhRab11B, which was previously demonstrated to be on the TGN (Mitra et al., 2007). The transformant cells expressing EhRab8A with the amino-terminal 3myc-tag were established and subjected to immunoblot analysis using anti-EhRab8A and anti-myc antibodies (Fig. 3A). Two bands were observed in the mock control using anti-EhRab8A antibody; the top (23.8 kDa, red asterisk) and bottom (22.8 kDa, black asterisk) bands likely corresponded to modified and non-modified endogenous EhRab8A. In myc-EhRab8A-expressing cells, four bands were observed; the two top bands (27.2 kDa, red cross; 26.4 kDa, black cross) corresponded to the exogenously expressed myc-tagged EhRab8A with or without modification(s), whereas the bottom two bands correspond to the endogenous EhRab8A. The nature of the modification remains unknown. We examined the nature (membrane-bound or soluble) of two forms of EhRab8A by differential centrifugation. The 22.8 and 26.4 kDa bands were recovered in the pellet fraction after centrifugation at 13,000 × g together with a transmembrane protein CPBF1 (Nakada-Tsukui et al., 2012), while the 23.8 and 27.2 kDa bands were found in the soluble fraction of centrifugation at 100,000 × g, together with a cytosolic soluble protein ICP1 (Sato et al., 2006) (Fig. 3B). The amount of exogenously expressed myc-EhRab8A protein was 2.1 ± 0.64-fold higher than that of the endogenous protein (Fig. 3A). An

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**Fig. 3. Subcellular fractionation of EhRab8A.**

A. Immunoblot analysis of endogenous (wild-type) and myc-EhRab8A detected by anti-EhRab8A (left) and anti-myc (right) antibodies. In mock cells, two bands corresponding to endogenous unmodified (a black asterisk) and modified EhRab8A (a red asterisk) were detected. In mock control, two bands corresponding to endogenous modified (a red asterisk) and myc-tagged modified EhRab8A (red cross) were detected.

B. Immunoblot analysis of EhRab8A in fractionated cell lysates. Lysates from myc-EhRab8A transformant cells were fractionated by centrifugation into the low-speed pellet (p13, the pellet fraction of 13,000 × g centrifugation), the high-speed pellet (p100, the pellet fraction of 100,000 × g centrifugation) and the supernatant fractions (s100, the supernatant fraction of 100,000 × g centrifugation). These fractions were subjected to immunoblot analysis using anti-EhRab8A, anti-CPBF1 (a membrane protein) and anti-ICP1 (a cytosolic protein) antibodies. Note that two bands corresponding to endogenous unmodified (a black asterisk) and myc-tagged unmodified EhRab8A (red cross) were detected.
myc-EhRab8A cells conducted using anti-EhRab8A and anti-myc antibodies revealed high colocalization (Pearson’s correlation coefficient: \( R=0.83 \)) (Fig. 4A), indicating that endogenous EhRab8A and exogenously expressed myc-EhRab8A were localized to the same organelle(s)/compartments. EhRab8A did not colocalize with EhRab11B, which was visualized with an anti-EhRab11B antibody (Mitra et al., 2007) (\( R=0.24 \), Fig. 4B). Furthermore, EhRab8A did not colocalize with Vps26, one of the retromer component that is involved in the recycling of receptors in the TGN and the endosomal compartment and well colocalized with the late endosomal marker EhRab7A (Nakada-Tsukui et al., 2005; Rojas et al., 2008; Cullen and Korswagen, 2012) (\( R=0.05 \), Fig. 4C).

Fig. 4. Lack of colocalization of exogenously expressed myc-EhRab8A and the trans-Golgi network markers.

A–C. Immunofluorescence imaging of exogenously expressed myc-EhRab8A, stained with anti-myc (green) and endogenous EhRab8A, stained with anti-EhRab8A (A, red), anti-EhRab11B (B, red) or anti-EhVps26 (C, red) antibodies. Histograms of the green and red intensities along the lines indicated in the islets on the bottom right of each image are shown at the bottom left of panels A–C. Major peaks of green, red and merged signals are depicted by arrows. The lengths of the lines are also shown. In the bottom right of panels, scatter plots showing the results of colocalization analyses are shown. \( R= \) Pearson’s correlation coefficient. Thick bars, 2 \( \mu m \).
ization of EhRab8A also clearly differed from that of EhRab7B and EhRabB, which is localized to lysosomes or the phagocytic mouth respectively (Rodriguez et al., 2000; Saito-Nakano et al., 2007) (Fig. S4). This observation was in contrast to other organisms (Knodler et al., 2010; Wang et al., 2012). In mammals, Rab10, which shares 70% amino acid identity with Rab8 (Chen et al., 1993; Pereira-Leal and Seabra, 2001) (Fig. S2), is known to localize to endosomes (e.g. in Madin–Darby canine kidney epithelial cells) (Babbee et al., 2006; Cardoso et al., 2010). In addition, Rab10 is localized to the ER in Xenopus laevis egg, COS-7 and HeLa cells (English and Voeltz, 2013); we next examined if EhRab8A localizes to the ER using antibodies raised against bacterial recombinant proteins of the ER luminal chaperone BiP (EHI_199890, 64% amino acid identity to human BiP, E-value = 0) (Munro and Pelham, 1986) and a component of the COPII vesicle, Sec13 (EHI_001050, 38% amino acid identity to human Sec13, E-value = 6e-46) (Barlowe et al., 1994). Monospecificities of the anti-BiP and anti-Sec13 antibodies were verified by immunoblot analyses of amoebic lysates (Fig. S3). An indirect immunofluorescence assay performed using the anti-BiP antibody revealed partial colocalization of myc-EhRab8A and BiP, suggesting that EhRab8A was localized to the ER or a compartment adjacent to the ER ($R = 0.7$, Fig. 5A). A subdomain of the ER, where COPII vesicle budding occurs, was named the ER exit site (ERES) (Jensen and Schekman, 2011), and it was visualized with the anti-Sec13 antibody. Sec13 did not colocalize with EhRab8A ($R = 0.15$, Fig. 5B). These indirect immunofluorescence assay data were also supported by the data of physical separation by two rounds of discontinuous Percoll gradient ultracentrifugation (Mi-ichi et al., 2009), followed by immunoblot assay, in which the distribution pattern of EhRab8A was similar to that of Sec13 (Fig. S5).

Overexpression of wild-type Rab8A enhanced phagocytosis, whereas a dominant-negative Rab8 mutant inhibited phagocytosis

Nucleotide-fixed state mutations can be engineered to alter the activity of Rab proteins (Stenmark et al., 1994).

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**Fig. 5.** Colocalization of exogenously expressed myc-EhRab8A and endoplasmic reticulum markers.

A and B. Immunofluorescence imaging of exogenously expressed myc-EhRab8A, stained with anti-myc (green) and the anti-EhBiP (A, red) or anti-EhSec13 (B, red) antibodies. Histograms of the green and red intensities along the lines indicated in the islets on the bottom right of each image are shown at the bottom left of panels A and B. Major peaks of green, red and merged signals are depicted by arrows. The lengths of the lines are also shown. In the bottom right of panels A and B, scatter plots showing the results of the colocalization analyses are shown. $R = $ Pearson’s correlation coefficient. Thick bars, 2 μm.
Two mutants of myc-EhRab8A, a GTP-fixed Q66L mutant and a GDP-fixed S21N mutant, were generated and introduced into the amoeba trophozoites. One of the two mutants, in which Gln in the second GTP-binding motif was replaced with Leu, was designed to have reduced GTPase activity and regarded as a constitutively active (GTP-fixed) mutant (Q66L) (Der et al., 1986; Stenmark et al., 1994). The other mutant possessing a Ser-to-Asn mutation in the first GTP-binding motif is assumed to possess a lower affinity for GTP than for GDP and functions as a constitutively negative (GDP-fixed) form (S21N) (Feig and Cooper, 1988; Stenmark et al., 1994).

Confocal fluorescence micrographs revealed that 40–60% of the trophozoites were stained with the anti-myc antibody (Fig. 6A). Localization of myc-EhRab8A Q66L mutant was similar to that of wild-type Rab8A and also well colocalized to BiP, whereas S21N mutant revealed dispersed cytosolic staining like the Rab8 SN mutant in other organisms (Walworth et al., 1989; Peranen et al., 1996) (Fig. S6). The localization of Q66L and S21N mutants is consistent with presumed characteristics of GTP-fixed and GDP-fixed mutants. We quantified the amount of fluorescein-labelled erythrocytes ingested by the trophozoites expressing either wild-type myc-

**Fig. 6.** Expression of EhRab8A GTP-bound mutant caused a defect in phagocytosis.
A. Immunofluorescence imaging of EhRab8A and erythrophagocytosis. Transformant trophozoites expressing myc-EhRab8A wild-type or myc-EhRab8A-Q66L mutant and mock transformant cells were mixed with PKH-labelled erythrocytes for 20 min at 37°C and reacted with an anti-myc antibody in an immunofluorescence assay.
B. Efficiency of erythrophagocytosis by the transformants expressing myc-EhRab8A wild type, myc-EhRab8A-Q66L or myc-EhRab8A-S21N mutant and mock transformant. The total fluorescence intensities of the PKH26-labelled erythrocytes ingested by a single trophozoite were measured, and the average of the total fluorescence intensities per trophozoite for each strain is shown. Note that the expression of myc-EhRab8A wild type and the myc-EhRab8A-Q66L mutant enhanced and reduced erythrophagocytosis respectively.
C. The population doubling time of myc-EhRab8A wild-type, myc-EhRab8A-S21N mutant and mock transformant cells. The correlation coefficients were calculated using Student’s t-test.
et al (Walworth completion of EhRab8A function like in other organisms and guanine nucleotide exchange is essential for the tosis caused by the expression of GDP-fixed mutant. This explanation for the observed slight increase of phagocy-
effectors and elicit phenotypes. Thus, we have no good compete with the intrinsic wild-type Rab for downstream
fixed mutant Rab, but not GDP-fixed mutant, is able to
Q66L mutant (Fig. 6C). In general, overexpressed GTP-
mutable caused no defect in growth, unlike GTP-locked
compared with the mock control, indicating that EhRab8A and EhRab7B are involved in progression of phagocytosis, suggesting that the ER and the PM are dynamically interconnected in E. histolytica (Marquie-
Markiewicz et al., 2011; Vaithilingam et al., 2012) (also see below).

The transport of PM receptors is mediated by EhRab8A
Among seven Rab proteins that we successfully silenced by gene silencing, EhRab8A and EhRab7B gene-
silencing strains showed a defect in phagocytosis. Interestingly, adhesion of EhRab7B gene-silencing strain to erythrocytes was comparable with that of mock transformant, indicating that EhRab8A and EhRab7B are involved in distinct stages of phagocytosis (our unpublished result). Surface biotinylation has shown that a few major surface proteins (200, 70 and 30 kDa) were apparently missing in the EhRab8A gene-silenced strain (Fig. 2C). Although their identity remains unknown, they are probably involved in the adherence to various extracellular prey and transported from the ER to the PM in an EhRab8A-dependent mechanism. The amoebic cell

Discussion
ER-localized EhRab8A is required for phagocytosis
In this study, we have shown using two genetic approaches that in E. histolytica unique ER-localized Rab8 is involved in phagocytosis. First, we have demonstrated that a specific repression of the EhRab8A gene expression by transcriptional gene silencing reduced amoebic adherence to and phagocytosis of prey and also surface presentation of a few PM proteins (Figs. 1 and 2). Further, we have also shown that overexpression of the wild type or GTP-fixed mutant of EhRab8A enhanced or reduced phagocytosis respectively (Fig. 6). Although statistically insignificant, expression of the GDP-fixed mutant caused a slight increase (~15%) on phagocytosis compared with the mock control. EhRab8A GDP-fixed mutant caused no defect in growth, unlike GTP-locked Q66L mutant (Fig. 6C). In general, overexpressed GTP-
fixed mutant Rab, but not GDP-fixed mutant, is able to
compete with the intrinsic wild-type Rab for downstream
effectors and elicit phenotypes. Thus, we have no good explanation for the observed slight increase of phagocy-
tosis caused by the expression of GDP-fixed mutant. This observation may indicate that the cycle of GTP hydrolysis and guanine nucleotide exchange is essential for the completion of EhRab8A function like in other organisms (Walworth et al., 1989; Peranen et al., 1996). Rab8 is a member of the highly conserved Rab subfamily in eukaryotes, including Metazoa (Caenorhabditis elegans and Nematostella vectensis), Chaozoa (Monosiga brevicollis), Fungi (Saccharomyces cerevisiae), Amoebozoa (Dictostelium discoideum), Viridiplantae (Arabidopsis thaliana), Stramenopiles (Phytophthora sojae), Ciliophora (Tetrahymena thermophila) and Schizopyrenida (Naegleria gruberi), but missing in Diplomonadida (Giardia lambia) and Trichomonadida (Trichomonas vaginalis) (Pereira-Leal and Seabra, 2001; Saito-Nakano et al., 2010; Diekmann et al., 2011). In mammals and yeasts, Rab8 is involved in trafficking from the TGN to the PM (Hutagalung and Novick, 2011). In free-living D. discoideum, Rab8 is localized to a contractile vacuole, which is a specialized osmoregulatory organelle involved in water expulsion (Essid et al., 2012). A line of evidence provided in this study suggests that EhRab8A plays a unique function in Entamoeba: EhRab8A is involved in the amoeba’s adherence to prey by mediating the transport of candidate receptor(s) to the PM (Fig. 2). Until now, many ER proteins including Rab1 have been identified in proteome analyses of macrophage phagosomes (Rogers and Foster, 2007; Gutierrez, 2013); the results suggested that the ER may supply membranes to the PM to engulf large foreign particles and to recruit major histocompatibility complex class I mole-
cules to phagosomes (Gagnon et al., 2002; Gueronprez et al., 2003). Although it remains to be debated whether the ER is involved in phagocytosis, more specifically the ER provides membrane to newly formed phagocytosis in E. histolytica, several ER proteins were identified from purified phagosomes by previous proteomic analyses in E. histolytica: calreticulin, BIP (Boettner et al., 2008) and COPII components (Marion et al., 2005). These data are consistent with the notion that the amoebic ER is involved in progression of phagocytosis. It has been previously shown that an ER luminal protein, calreticulin, is present on the amoebic cell surface and involved in erythrophagocytosis, suggesting that the ER and the PM are probably involved in the adherence to various extracellular prey and transported from the ER to the PM in an EhRab8A-dependent mechanism. The amoebic cell
surface proteome, comprising nearly 700 proteins (Biller et al., 2014), possibly contains these potential surface receptors. Our attempt to identify and characterize these potential surface receptors is underway. It is noteworthy that the transport of at least three proteins, the heavy and intermediate subunits of the Gal/GalNAc lectin and KERP1, was not mediated by EhRab8A as their surface expression remained unchanged by EhRab8A gene silencing (Fig. S1). The light subunit of Gal/GalNAc lectin (30–35 kDa) (Petri et al., 1989), which is coded by five independent genes (Bracha et al., 2007), is an alternative candidate for the 30 kDa surface protein transported by EhRab8A.

EhRab8A may be involved in a non-conventional traffic pathway from the ER to the PM

The absence of EhRab8A at the ERES (Fig. 4C) was noteworthy. In general, cargo proteins transported to the Golgi are recognized by p24 families of proteins and a component of COPII, Sec24 protein, and then packed into the COPII vesicles in the ERES (Demmel et al., 2011; Herzig et al., 2012). During formation of the COPII vesicle, a member of the Arf family of GTPases called Sar1, together with the p24 families of proteins, plays a pivotal role in cargo sorting into COPII vesicles in the ER (Vendetti et al., 2014). Thus, our data suggested that the cargo sorting in a pathway regulated by EhRab8A differed from the canonical mechanism for the COPII vesicle formation mediated by the p24 family and Sec24.

Human Rab10 is closely related to Rab8 and localized to the ER (70% amino acid identity, Fig. S2) and forms a specialized domain linked to phospholipid synthesis involving phosphatidylinositol synthase (PIS) and ethanolamine phosphotransferase 1 (CEPT1) (English and Voeltz, 2013). It has also been shown that expression of a mutant Rab10 or depletion of endogenous Rab10 reduces ER tubular extension, likely attributable to a disturbance of the Rab10-associated enzymes involved in phospholipid biosynthesis in the ER subdomain (English and Voeltz, 2013). The E. histolytica genome encodes both PIS and CEPT1 (EH1_069630 for CDP-DAG inositol 3-phosphatidyltransferase; EH1_148580 and EH1_152340 for ethanolamine/choline kinase). Thus, it is plausible that EhRab8A regulates the transport of PM proteins via the lipid biosynthesis. Our preliminary co-immunoprecipitation of EhRab8A identified several lipid-metabolizing enzymes, which also supports a premise that EhRab8A is involved in lipid/phospholipid biosynthesis and transfer.

Lack of the Rab11–Rab8 cascade for transport from the TGN to the PM in E. histolytica

In the mammalian secretory pathway, Rab11 functions upstream of Rab8. The GTP-bound active form of Rab11 binds its effector molecule, Rabin8, which is a guanine nucleotide exchange factor (GEF) of Rab8 (Hattulla et al., 2002). Consistently, partial colocalization of Rab8 and Rab11 in the TGN has been reported in mammalian cells (Hattulla et al., 2006; Knodler et al., 2010). This Rab cascade, comprising Ypt32p and Sec2p, homologues of Rab11 and Rabin8, respectively, is also conserved in the budding yeast (Walch-Solimena et al., 1997). In contrast, in E. histolytica, EhRab8A and EhRab11B were not colocalized (Fig. 4B), indicating that EhRab8A and EhRab11B regulate non-overlapping steps of the same pathway or independent pathways. We have previously reported that overexpression of EhRab11B resulted in massive mis-secretion of cysteine proteases, suggesting that EhRab11B is the primary regulator of cysteine protease transport from the TGN to the PM in E. histolytica (Mitra et al., 2007). Lack of the Rab11–Rab8 cascade in E. histolytica is also consistent with the finding that Sec2, Rab8 GEF, is only conserved in Opisthokonts, with exceptions of Dipterans and Microsporidia, and absent in Amoeboida (Elias, 2008). These data suggest that the role and its regulation of Rab8 in Entamoeba are highly divergent from that in Opisthokonts. Identification and characterization of effector proteins (and upstream GEF) of EhRab8A, which is underway, may help elucidate the evolution of Rab8-mediated membrane trafficking in divergent eukaryotes.

Experimental procedures

Culture

Trophozoites of the E. histolytica strain HM-1: IMSS cl-6 were cultured axenically at 35°C in 13 × 100 mm screw-capped Pyrex glass tubes or plastic culture flasks in BI-S-33 medium, as previously described (Diamond et al., 1972; 1978).

Establishment of an EhRab8A gene-silenced strain, a 3myc-tagged EhRab8-expressing strain and strains expressing dominant-active and dominant-negative EhRab8A mutants

A plasmid for the gene silencing of EhRab8A was constructed as follows. A 480-bp-long 5′ end of the EhRab8A protein-coding region was amplified by PCR from cDNA using the following oligonucleotides: 5′-AAA AGG CCT TCG GAG AAC GAT TCA ACA ATT-3′ and 5′-GCC GAG CTC ACA GTC ATC AAC TTT TAA TGA-3′ (the Stul and SacI restriction sites are underlined). The PCR-amplified DNA fragment was digested with Stul and SacI and ligated into Stul and SacI double-digested pSAP2-Gumma (Mi-ichi et al., 2011) to produce pSAP2-EhRab8A. The EhRab8A gene-silenced strain was established by transfection of the G3 strain (Bracha et al., 2006) by liposome-mediated transfection, as previously described (Nozaki et al., 1999; Saito-Nakano et al., 2004); stable transformants were cultured in the medium containing 10 μg ml −1 Geneticin (Life Tech Orienta).
Moreover, we established a control mock transformant by transfection of the G3 strain with pSAP2-Gunma.

A plasmid was constructed as follows for the creation of a transformant wherein EhRab8A, with the 3myc-tag at the amino terminus, was expressed. A 600 bp DNA fragment containing the EhRab8A-coding sequence was amplified by PCR from cDNA with the following oligonucleotides: 5′-GAA GAG GAT TCT ATG TCG GAG AAG GAT TCA ACA-3′ and 5′-GGT CAA TCT TGA ACA TCC AGT TGA TCC AGT-3′ (the XhoI restriction sites are underlined). The amplified fragment was cloned into Xhol-digested pKT-3M (Saito-Nakano et al., 2004) with an Infusion cloning kit (Clontech). Plasmids that expressed EhRab8A S21N (GDP-bound dominant-negative) and Q66L (GTP-bound dominant-active) mutants were constructed by PCR-mediated mutagenesis (Landt et al., 1990) using a PrimeSTAR Mutagenesis Basal Kit (Takara). These plasmids were introduced into trophozoites as described earlier.

**Quantitative real-time PCR**

mRNA expressions of EhRab8A and EhRab8B were analysed by quantitative real-time PCR analyses, essentially as previously described (Gilchrist et al., 2006; Saito-Nakano et al., 2007), with some modifications. RNA polymerase II served as an internal control (GenBank accession number XP_649091). The primers used were as follows: 5′-ATT CCA TTC TTA GAA ACA TCT GCC AAA AAT-3′ and 5′-GAA GAA GGA GGA AAT GTG ATT AAC AAC ATC-3′ (EhRab8A); 5′-GAG CTT GCT GAT AAA TTA GGT ATT CTG TTC-3′ and 5′-ACA ACA GCC ACT TTG ACC TCT TTA TGA AGA-3′ (EhRab8B); and 5′-GAT CCA ACA TAT CCT AAA ACA ACA-3′ and 5′-TCA ATT TTC TTA CCC GTC TTC-3′ (RNA polymerase II). The following parameters were used: an initial step of denaturation at 95°C for 9 min, followed by 40 cycles of denaturation at 94°C for 30 s, annealing at 50°C for 30 s and extension at 65°C for 1 min. A final step at 95°C for 9 s, 60°C for 9 s and 95°C for 9 s was used to remove the primer dimers.

**Production of recombinant EhRab8A, EhBiP, EhSec13 and EhRab11B**

In order to generate a plasmid expressing the maltose-binding protein-fused EhRab8A-recombinant protein, the full-length EhRab8A open reading frame was inserted into the pMAL-c2X vector (New England Biolabs, Ipswich, MA, USA). Further, to generate a plasmid expressing glutathione-S-transferase-fused BiP or Sec13, the C-terminal domain of BiP (amino acids 435–661) or full-length Sec13 was inserted into the pGEX-6p1 vector (GE Healthcare). The full length of EhRab11B was inserted into the pCold vector (Takara) for expressing the histidine-tagged recombinant EhRab11B. Recombinant proteins were expressed using the *E. coli* strain BL21 (DE3) and purified according to the manufacturer’s instructions.

**Antibodies**

Antiserum was commercially raised against recombinant *EhRab8A*, *EhBiP*, *EhSec13* or *EhRab11B* in rabbits (Eurofins Genomics, Japan). The *EhRab8A* IgG was further purified with HiTrap NHS-activated HP column (Amersham) coupled with purified recombinant maltose-binding protein-conjugated-*EhRab8A* according to the manufacturer’s instruction. The antiserum was purified using a HiTrap protein G kit (GE Healthcare).

**Phagocytosis assay**

Phagocytosis was assayed by either flow cytometry (FACS Calibur) or microscopy. The flow cytometry assay for amoebic ingestion was adapted from the method previously described by Huston et al. (2003), with some modifications. ImmunoFluoSpheres (2 μm of carboxylated Nile Red-labelled beads, Invitrogen), PKH26 red fluorescent-labelled (Sigma) erythrocytes and fluorescein-conjugated *E. coli* K-12 strain BioParticles (Invitrogen) were incubated with 1 × 10^5* amoebic trophozoites in 200 μl of BIS medium in a well of a 12-well plate at 37°C for 10–60 min. Following the incubation, the amoebae and beads or cells were resuspended in 2% D-galactose in ice-cold phosphate-buffered saline (PBS) and analysed by flow cytometry. The amoebae were distinguished from beads, erythrocytes and *E. coli* cells on the basis of differences in the forward-scatter and side-scatter signal characteristics and background fluorescence. Efficiency of the amoebic ingestion of targets was determined by the percentages of amoebae with fluorescence levels above the background. For the microscopy assay to examine the phagocytosis efficiency, the myc-EhRab8A dominant-negative-mutant-expressing cells were attached to the slide glass and engulfed with PKH26-labelled erythrocytes for 20 min. The samples were fixed, stained with an anti-myc antibody and visualized with an anti-mouse Alexa 488 antibody, as described in the following indirect immunofluorescence assay. The fluorescent intensity of the PKH26 signal in the trophozoites stained with the anti-myc antibody was captured on a Carl Zeiss LSM780 confocal laser scanning microscope, and the images were analysed with ZEN software (Zeiss).

**Adherence assay**

*Entamoeba histolytica* adherence was assayed as previously described (Huston et al., 2003) with some modifications. Approximately 1 × 10^5 amoebae and 1 × 10^7 erythrocytes were resuspended in 1 ml Opti-MEM I medium (Life Technologies) supplemented with 5 mg ml^−1^ of cysteine and 1 mg ml^−1^ of ascorbic acid (pH 6.8), centrifuged (440 × g, 5 min, 4°C) and incubated for 30 min on ice. After the incubation, the supernatant was poured off, and the pellet was resuspended in 100 μl of transfection medium. The sample was then examined under a microscope.

**Surface biotinylation**

Approximately 1 × 10^6 live *E. histolytica* trophozoites were biotinylated in 500 μl of Opti-MEM, pH 8.0, for 15 min at 4°C with EZ-Link Sulfo-NHS-Biotin (Pierce), as previously described (Berro et al., 2007). After the suspension, the cells were centrifuged at 500 × g for 5 min in 0.5 ml of cold Dulbecco’s PBS with 0.1% lysine that
was added to the cell pellet, and the cells were further washed two times with PBS. Next, the cells were lysed by 1× SDS sample buffer and subjected to immunoblot analysis with avidin–HRP and SYPRO Ruby (Life Technologies) staining.

**Subcellular fractionation**

The accessibility by proteinase K assay was performed as reported previously with some modifications (Saito-Nakano et al., 2007). Approximately $3 \times 10^6$ amoeba cells were washed with cold PBS containing 2% glucose, resuspended in homogenization buffer (250 mM sucrose, 50 mM Tris, pH 7.5, 50 mM NaCl, 0.1 mg ml$^{-1}$ of E-64) and homogenized on ice with 30 strokes by a Dounce homogenizer with a tight-fitting pestle. After unbroken cells were removed by centrifugation at 400 × g for 2 min, the supernatant was centrifuged at 13,000 × g at 4°C for 10 min to obtain the pellet (p13) and supernatant (s13) fractions. The s13 fraction was further separated by centrifugation at 100,000 × g at 4°C for 1 h to obtain soluble (s100) fractions. These fractions were subjected to immunoblot analyses with anti-EhRab8A, anti-CPBF1 (Nakada-Tsukui et al., 2012) or anti-ICP1 antibodies (Sato et al., 2006).

**Confocal microscopy**

An indirect immunofluorescence assay was conducted, essentially as previously described (Saito-Nakano et al., 2004). Trophozoites were transferred to 8 mm round wells on a slide glass, fixed, permeabilized and reacted with an anti-myc 9E10 monoclonal antibody (Santa Cruz Biotech) to detect myc-tagged EhRab8A wild type and myc-tagged EhRab8A-GDP and GTP mutants, anti-EhRab11B (this study), anti-Vps26 (Nakada-Tsukui et al., 2005), anti-BIP (this study), anti-Sec13 (this study) polyconal rabbit sera and the anti-heavy (clone 7F4) and intermediate (clone EH3015) subunits of Gal/GalNac lectin, the last two of which were a gift from Nancy Guillén, William A. Petri Jr. for the antibody against the heavy subunit of the Gal/GalNAc lectin, Hiroshi Tachibana for the antibody against the intermediate subunit of the Gal/GalNAc lectin and Nancy Guillén for the antibody against the KERP1. We also thank Eiko Nakasone, Kazuo Ebine, Herbert J. Santos and Yoko Chiba for technical assistance and all members of our laboratory for valuable discussions.

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**References**

Babey, C.M., Ahktar, N., Wang, E., Chen, C.C., Grant, B.D., and Dunn, K.W. (2006) Rab10 regulates membrane transport through early endosomes of polarized Madin–Darby canine kidney cells. *Mol Biol Cell* 17: 3156–3175.

Barlowe, C., Orci, L., Yeung, T., Hosobuchi, M., Hamamoto, S., Salama, N., et al. (1994) COP11: a membrane coat formed by Sec proteins that drive vesicle budding from the endoplasmic reticulum. *Cell* 77: 895–907.

Beck, D.L., Boettner, D.R., Dragulev, B., Ready, K., Nozaki, T., and Petri, W.A., Jr. (2005) Identification and gene expression analysis of a large family of transmembrane kinases related to the Gal/GalNAc lectin in *Entamoeba histolytica*. *Eukaryot Cell* 4: 722–732.

Berro, R., de la Fuente, C., Klase, Z., Kehn, K., Parvin, L., Pumfrey, A., et al. (2007) Identifying the membrane proteome of HIV-1 latently infected cells. *J Biol Chem* 282: 8207–8218.

Billir, L., Matthiesen, J., Kuhne, V., Lotter, H., Handal, G., Nozaki, T., et al. (2014) The cell surface proteome of *Entamoeba histolytica*. *Mol Cell Proteomics* 13: 132–144.

Boettner, D.R., Huston, C.D., Linford, A.S., Buss, S.N., Houpt, E., Sherman, N.E., and Petri, W.A., Jr. (2008) *Entamoeba histolytica* phagocytosis of human erythrocytes involves PATMK, a member of the transmembrane kinase family. *PLoS Pathog* 4: e8.

Boettner, D.R., Huston, C.D., Sullivan, J.A., and Petri, W.A., Jr. (2005) *Entamoeba histolytica* and *Entamoeba dispar* utilize externalized phosphatidylserine for recognition and phagocytosis of erythrocytes. *Infect Immun* 73: 3422–3430.

Bracha, R., Nuchamowitz, Y., Anbar, M., and Mirelman, D. (2006) Transcriptional silencing of multiple genes in trophozoites of *Entamoeba histolytica*. *PLoS Pathog* 2: e48.

Bracha, R., Nuchamowitz, Y., Wender, N., and Mirelman, D. (2007) Transcriptional gene silencing reveals two distinct groups of *Entamoeba histolytica* Gal/GalNac-lectin light subunits. *Eukaryot Cell* 6: 1758–1765.

Buss, S.N., Hamano, S., Vidrich, A., Evans, C., Zhang, Y., Crasta, O.R., et al. (2010) Members of the *Entamoeba histolytica* transmembrane kinase family play non-redundant roles in growth and phagocytosis. *Int J Parasitol* 40: 833–843.

Cardoso, C.M., Jordao, L., and Vieira, O.V. (2010) Rab10 regulates phagosome maturation and its overexpression rescues *Mycobacterium*-containing phagosomes maturation. *Traffic* 11: 221–235.

Chen, Y.T., Holcomb, C., and Moore, H.P. (1993) Expression and localization of two low molecular weight GTP-binding proteins, Rab8 and Rab10, by epitope tag. *Proc Natl Acad Sci U S A* 90: 6508–6512.

Cheng, X.J., Hughes, M.A., Huston, C.D., Loftus, B., Gilchrist, C.A., Lockhart, L.A., et al. (2001) Intermediate subunit of the Gal/GalNac lectin of *Entamoeba histolytica* is a member of a gene family containing multiple CXXC sequence motifs. *Infect Immun* 69: 5892–5898.

Cheng, X.J., Tsukamoto, H., Kameda, Y., and Tachibana, H. (1998) Identification of the 150-kDa surface antigen of *Entamoeba histolytica* as a galactose- and N-acetyl-d-galactosamine-inhibitable lectin. *Parasitol Res* 84: 632–639.

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Cullen, P.J., and Korswagen, H.C. (2012) Sorting nexins provide diversity for retromer-dependent trafficking events. Nat Cell Biol 14: 29–37.

Demmel, L., Melak, M., Kotisch, H., Fendos, J., Reipert, S., and Warren, G. (2011) Differential selection of Golgi proteins by COPII Sec24 isoforms in procyclic Trypanosoma brucei. Traffic 12: 1575–1591.

Der, C.J., Finkel, T., and Cooper, G.M. (1986) Biological and biochemical properties of human rasH genes mutated at codon 61. Cell 44: 167–176.

Diamond, L.S., Harlow, D.R., and Cunnick, C.C. (1978) A new medium for the axenic cultivation of Entamoeba histolytica and other Entamoeba. Trans R Soc Trop Med Hyg 72: 431–432.

Diamond, L.S., Mattern, C.F., and Bartgis, I.L. (1972) Viruses of Opisthokonta and the Archaeplastida. Sec2 and PRONE: candidate synapomorphies for the Sec2 and PRONE: candidate synapomorphies for the cell biologist. Mol Biol Evol 19: 169–178.

Essid, M., Gopaldass, N., Yoshida, K., Merrifield, C., and Guermonprez, P., Saveanu, L., Kleijmeer, M., Davoust, J., Van Endert, P., and Amigorena, S. (2003) ER–phagosome fusion defines an MHC class I cross-presentation compartment in dendritic cells. Nature 425: 397–402.

Gagnon, E., Duclos, S., Rondeau, C., Chevet, E., Cameron, P.H., Steele-Mortimer, O., et al. (2002) Endoplasmic reticulum-mediated phagocytosis is a mechanism of entry into macrophages. Cell 110: 119–131.

Gilchrist, C.A., Houpt, E., Trapanzde, N., Fei, Z., Crasta, O., Asghahrour, A., et al. (2006) Impact of intestinal colonization and invasion on the Entamoeba histolytica transcriptome. Mol Biochem Parasitol 147: 163–176.

Guerronprez, P., Saveanu, L., Kleijmeer, M., Davoust, J., Van Endert, P., and Amigorena, S. (2003) ER–phagosome fusion defines an MHC class I cross-presentation compartment in dendritic cells. Nature 425: 397–402.

Guillen, N. (2014) Infection biology: nibbled to death. Nature 508: 462–463.

Gutierrez, M.G. (2013) Functional role(s) of phagosomal Rab GTPases. Small GTPases 4: 148–158.

Hattula, K., Furuhjelm, J., Arffman, A., and Peranen, J. (2002) A Rab8-specific GDP/GTP exchange factor is involved in actin remodeling and polarized membrane transport. Mol Biol Cell 13: 3268–3280.

Hattula, K., Furuhjelm, J., Tikkanen, J., Tanhuanpaa, K., Laakkonen, P., and Peranen, J. (2006) Characterization of the Rab8-specific membrane traffic route linked to promotion formation. J Cell Sci 119: 4866–4877.

Herzig, Y., Sharpe, H.J., Elbaz, Y., Munro, S., and Schuldiner, M. (2012) A systematic approach to pair secretory cargo receptors with their cargo suggests a mechanism for cargo selection by Env14. PLoS Biol 10: e1001329.

Huber, L.A., Pimplikar, S., Parton, R.G., Virta, H., Zerial, M., and Simons, K. (1993) Rab8, a small GTPase involved in vesicular traffic between the TGN and the basolateral plasma membrane. J Cell Biol 123: 35–45.

Huston, C.D., Boettner, D.R., Miller-Sims, V., and Petri, W.A., Jr. (2003) Apoptotic killing and phagocytosis of host cells by the parasite Entamoeba histolytica. Infect Immun 71: 964–972.

Hutagalung, A.H., and Novick, P.J. (2011) Role of Rab GTPases in membrane traffic and cell physiology. Physiol Rev 91: 119–149.

Jensen, D., and Schekman, R. (2011) COPII-mediated vesicle formation at a glance. J Cell Sci 124: 1–4.

Juez, P., Sanchez-Lopez, R., Stock, R.P., Olvera, A., Ramos, M.A., and Alagon, A. (2001) Characterization of the EhRab8 gene, a marker of the late stages of the secretory pathway of Entamoeba histolytica. Mol Biochem Parasitol 116: 223–228.

Juarez-Hernandez, L.J., Garcia-Perez, R.M., Salas-Casas, A., Garcia-Rivera, G., Orozco, E., and Rodriguez, M.A. (2013) Entamoeba histolytica: the over expression of a mutated EhRab8 protein produces a decrease of in vitro and in vivo virulence. Exp Parasitol 133: 339–345.

Kirchen, J.M., and Ravichandran, K.S. (2008) Phagosome maturation: going through the acid test. Nat Rev Mol Cell Biol 9: 781–795.

Knoedler, A., Feng, S., Zhang, J., Zhang, X., Das, A., Peranen, J., and Guo, W. (2010) Coordination of Rab8 and Rab11 in primary ciliogenesis. Proc Natl Acad Sci U S A 107: 6346–6351.

Landt, O., Grunert, H.P., and Hahn, U. (1990) A general method for rapid site-directed mutagenesis using the polymerase chain reaction. Gene 96: 125–128.

MacFarlane, R.C., and Singh, U. (2007) Identification of an Entamoeba histolytica serine-, threonine-, and isoluecine-rich protein with roles in adhesion and cytotoxicity. Eukaryot Cell 6: 2139–2146.

Mann, B.J., Torian, B.E., Vedvick, T.S., and Petri, W.A., Jr. (1991) Sequence of a cysteine-rich galactose-specific lectin of Entamoeba histolytica. Proc Natl Acad Sci U S A 88: 3248–3252.

Marion, S., Laurent, C., and Guillen, N. (2005) Signalization and cytoskeleton activity through myosin IB during the early steps of phagocytosis in Entamoeba histolytica: a proteomic approach. Cell Microbiol 7: 1504–1518.
Okada, M., and Nozaki, T. (2006) New insights into molecular analysis of uropods in the pathogen Entamoeba histolytica. *PLoS Negl Trop Dis* 5: e1002.

McGugan, G.C., Jr., and Temesvari, L.A. (2003) Characterization of a Rab11-like GTPase, EhRab11, of *Entamoeba histolytica*. *Mol Biochem Parasitol* 129: 137–146.

Mi-ichi, F., Abu Yousuf, M., Nakada-Tsukui, K., and Nozaki, T. (2009) Mitosomes in *Entamoeba histolytica* contain a sulfite activation pathway. *Proc Natl Acad Sci U S A* 106: 21731–21736.

Mi-ichi, F., Makiuchi, T., Furukawa, A., Sato, D., and Nozaki, T. (2011) Sulfate activation in mitosomes plays an important role in the proliferation of *Entamoeba histolytica*. *PLoS Negl Trop Dis* 5: e1263.

Mitra, B.N., Saito-Nakano, Y., Nakada-Tsukui, K., Sato, D., and Nozaki, T. (2007) Rab11B small GTPase regulates secretion of cysteine proteases in the enteric protozoan parasite *Entamoeba histolytica*. *Cell Microbiol* 9: 2112–2125.

Munro, S., and Pelham, H.R. (1986) An Hsp70-like protein in the ER: identity with the 78 kd glucose-regulated protein and immunoglobulin heavy chain binding protein. *Cell* 46: 291–300.

Nakada-Tsukui, K., Okada, H., Mitra, B.N., and Nozaki, T. (2009) Phosphatidylinositol-phosphates mediate cytoskeleton reorganization during phagocytosis via a unique modular protein consisting of RhoGEF/DH and FYVE domains in the parasitic protozoan *Entamoeba histolytica*. *Cell Microbiol* 11: 1471–1491.

Nakada-Tsukui, K., Saito-Nakano, Y., Ali, V., and Nozaki, T. (2005) A retromerlike complex is a novel Rab7 effector that is involved in the transport of the virulence factor cysteine protease in the enteric protozoan parasite *Entamoeba histolytica*. *Mol Cell Biol* 16: 5294–5303.

Nakada-Tsukui, K., Saito-Nakano, Y., Husain, A., and Nozaki, T. (2010) Conservation and function of Rab small GTPases in *Entamoeba*: annotation of *E. invadens* Rab and its use for the understanding of *Entamoeba* biology. *Exp Parasitol* 126: 337–347.

Nakada-Tsukui, K., Tsuboi, K., Furukawa, A., Yamada, Y., and Nozaki, T. (2012) A novel class of cysteine protease receptors that mediate lysosomal transport. *Cell Microbiol* 14: 1299–1317.

Nozaki, T., Asai, T., Sanchez, L.B., Kobayashi, S., Nakazawa, M., and Takeuchi, T. (1999) Characterization of the gene encoding serine acetyltransferase, a regulated enzyme of cysteine biosynthesis from the protist parasites *Entamoeba histolytica* and *Entamoeba dispar*. Regulation and possible function of the cysteine biosynthetic pathway in *Entamoeba*. *J Biol Chem* 274: 32445–32452.

Okada, M., Huston, C.D., Mann, B.J., Petri, W.A., Jr., Kita, K., and Nozaki, T. (2005) Proteomic analysis of phagocytosis in the enteric protozoan parasite *Entamoeba histolytica*. *Eukaryot Cell* 4: 827–831.

Okada, M., Huston, C.D., Oue, M., Mann, B.J., Petri, W.A., Jr., Kita, K., and Nozaki, T. (2006) Kinetics and strain variation of phagosome proteins of *Entamoeba histolytica* by proteomic analysis. *Mol Biochem Parasitol* 145: 171–183.

Okada, M., and Nozaki, T. (2006) New insights into molecular mechanisms of phagocytosis in *Entamoeba histolytica* by proteomic analysis. *Arch Med Res* 37: 244–252.

Peranen, J., Auvinen, P., Virta, H., Wepf, R., and Simons, K. (1996) Rab8 promotes polarized membrane transport through reorganization of actin and microtubules in fibroblasts. *J Cell Biol* 135: 153–167.

Pereira-Leal, J.B., and Seabra, M.C. (2001) Evolution of the Rab family of small GTP-binding proteins. *J Mol Biol* 313: 889–901.

Petri, W.A., Jr., Chapman, M.D., Snodgrass, T., Mann, B.J., Broman, J., and Ravidin, J.I. (1989) Subunit structure of the galactose and N-acetyl-D-galactosamine-inhibitable adherence lectin of *Entamoeba histolytica*. *J Biol Chem* 264: 3007–3012.

Petri, W.A., Jr., Jackson, T.F., Gathiram, V., Kress, K., Saffer, L.D., Snodgrass, T.L., et al. (1990) Pathogenic and nonpathogenic strains of *Entamoeba histolytica* can be differentiated by monoclonal antibodies to the galactose-specific adherence lectin. *Infect Immun* 58: 1802–1806.

Ralston, K.S. (2015) Chew on this: amoebic trogocytosis and host cell killing by *Entamoeba histolytica*. *Trends Parasitol* 31: 442–452.

Ralston, K.S., and Petri, W.A., Jr. (2011) Tissue destruction and invasion by *Entamoeba histolytica*. *Trends Parasitol* 27: 254–263.

Ralston, K.S., Solga, M.D., Mackey-Lawrence, N.M., Somlata, Bhattacharya, A., and Petri, W.A., Jr. (2014) Trogocytosis by *Entamoeba histolytica* contributes to cell killing and tissue invasion. *Nature* 508: 526–530.

Rodriguez, M.A., Garcia-Perez, R.M., Garcia-Rivera, G., Lopez-Reyes, I., Mendoza, L., Ortiz-Navarrete, V., and Orozco, E. (2000) An *Entamoeba histolytica* rab-like encoding gene and protein: function and cellular location. *Mol Biochem Parasitol* 108: 199–206.

Rogers, L.D., and Foster, L.J. (2007) The dynamic phagosomal proteome and the contribution of the endoplasmic reticulum. *Proc Natl Acad Sci U S A* 104: 18520–18525.

Rojas, R., van Vlijmen, T., Mardones, G.A., Prabhu, Y., Rojas, A.L., Mohammed, S., et al. (2008) Regulation of retromer recruitment to endosomes by sequential action of Rab5 and Rab7. *J Cell Biol* 183: 513–526.

Saito-Nakano, Y., Loftus, B.J., Hall, N., and Nozaki, T. (2005) The diversity of Rab GTPases in *Entamoeba histolytica*. *Exp Parasitol* 110: 244–252.

Saito-Nakano, Y., Mita, B.N., Nakada-Tsukui, K., Sato, D., and Nozaki, T. (2007) Two Rab7 isoforms, EhRab7A and EhRab7B, play distinct roles in biogenesis of lysosomes and phagosomes in the enteric protozoan parasite *Entamoeba histolytica*. *Cell Microbiol* 9: 1796–1808.

Saito-Nakano, Y., Nakahara, T., Nakano, K., Nozaki, T., and Numata, O. (2010) Marked amplification and diversification of products of ras genes from rat brain, Rab GTPases, in the ciliates *Tetrahymena thermophila* and *Paramecium tetraurelia*. *J Eukaryot Microbiol* 57: 389–399.

Saito-Nakano, Y., Yasuda, T., Nakada-Tsukui, K., Leippe, M., and Nozaki, T. (2004) *EhRab5*-associated vacuoles play a unique role in phagocytosis of the enteric protozoan parasite *Entamoeba histolytica*. *J Biol Chem* 279: 49497–49507.

Salminen, A., and Novick, P.J. (1987) A ras-like protein is required for a post-Golgi event in yeast secretion. *Cell* 49: 527–538.
Santi-Rocca, J., Weber, C., Guignon, G., Sismeiro, O., Coppee, J.Y., and Guillen, N. (2008) The lysine- and glutamic acid-rich protein KERP1 plays a role in Entamoeba histolytica liver abscess pathogenesis. Cell Microbiol 10: 202–217.

Sato, D., Nakada-Tsukui, K., Okada, M., and Nozaki, T. (2006) Two cysteine protease inhibitors, EhICP1 and 2, localized in distinct compartments, negatively regulate secretion in Entamoeba histolytica. FEBS Lett 580: 5306–5312.

Seigneur, M., Mounier, J., Prevost, M.C., and Guillen, N. (2005) A lysine- and glutamic acid-rich protein, KERP1, from Entamoeba histolytica binds to human enterocytes. Cell Microbiol 7: 569–579.

Shrimal, S., Bhattacharya, S., and Bhattacharya, A. (2010) Serum-dependent selective expression of EhTMKB1-9, a member of Entamoeba histolytica B1 family of transmembrane kinases. PLoS Pathog 6: e1000929.

Stanley, S.L., Jr., Becker, A., Kunz-Jenkins, C., Foster, L., and Li, E. (1990) Cloning and expression of a membrane antigen of Entamoeba histolytica possessing multiple tandem repeats. Proc Natl Acad Sci U S A: 4976–4980.

Stenmark, H. (2009) Rab GTPases as coordinators of vesicle traffic. Nat Rev Mol Cell Biol 10: 513–525.

Stenmark, H., Parton, R.G., Steele-Mortimer, O., Lutcke, A., and Zerial, M. (1994) Inhibition of rab5 GTPase activity stimulates membrane fusion in endocytosis. EMBO J 13: 1287–1296.

Stuart, L.M., and Ezekowitz, R.A. (2005) Phagocytosis: elegant complexity. Immunity 22: 539–550.

Trost, M., English, L., Lemieux, S., Courcelles, M., Desjardins, M., and Thibault, P. (2009) The phagosomal proteome in interferon-gamma-activated macrophages. Immunity 30: 143–154.

Urwyler, S., Nyfeler, Y., Ragaz, C., Lee, H., Mueller, L.N., Aebbersold, R., and Hilbi, H. (2009) Proteome analysis of Legionella vacuoles purified by magnetic immunoseparation reveals secretory and endosomal GTPases. Traffic 10: 76–87.

Vaitilingam, A., Teixeira, J.E., Miller, P.J., Heron, B.T., and Huston, C.D. (2012) Entamoeba histolytica cell surface calreticulin binds human c1q and functions in amebic phagocytosis of host cells. Infect Immun 80: 2008–2018.

Venditti, R., Wilson, C., and De Matteis, M.A. (2014) Exiting the ER: what we know and what we don’t. Trends Cell Biol 24: 9–18.

Vieira, O.V., Botelho, R.J., and Grinstein, S. (2002) Phagosome maturation: aging gracefully. Biochem J 366: 689–704.

Walch-Solimena, C., Collins, R.N., and Novick, P.J. (1997) Sec2p mediates nucleotide exchange on Sec4p and is involved in polarized delivery of post-Golgi vesicles. J Cell Biol 137: 1495–1509.

Walworth, N.C., Goud, B., Kabcenell, A.K., and Novick, P.J. (1989) Mutational analysis of SEC4 suggests a cyclical mechanism for the regulation of vesicular traffic. EMBO J 8: 1685–1693.

Wang, J., Morita, Y., Mazelova, J., and Deretic, D. (2012) The Arf GAP ASAP1 provides a platform to regulate Arf4- and Rab11-Rab8-mediated ciliary receptor targeting. EMBO J 31: 4057–4071.

Welter, B.H., Powell, R.R., Leo, M., Smith, C.M., and Temesvari, L.A. (2005) A unique Rab GTPase, EhRabA, is involved in motility and polarization of Entamoeba histolytica cells. Mol Biochem Parasitol 140: 161–173.

Welter, B.H., and Temesvari, L.A. (2004) A unique Rab GTPase, EhRabA, of Entamoeba histolytica, localizes to the leading edge of motile cells. Mol Biochem Parasitol 135: 185–195.

Welter, B.H., Powell, R.R., Leo, M., Smith, C.M., and Temesvari, L.A. (2005) A unique Rab GTPase, EhRabA, is involved in motility and polarization of Entamoeba histolytica cells. Mol Biochem Parasitol 140: 161–173.

Welter, B.H., and Temesvari, L.A. (2004) A unique Rab GTPase, EhRabA, of Entamoeba histolytica, localizes to the leading edge of motile cells. Mol Biochem Parasitol 135: 185–195.

Zhang, H., Alrarnini, H., Tran, V., and Singh, U. (2011) Nuclear-localized antisense small RNAs with 5′-polyphosphate termini regulate long term transcriptional gene silencing in Entamoeba histolytica G3 strain. J Biol Chem 286: 44467–44479.

Zhao, Y., Zhang, W., and Kho, Y. (2004) Proteomic analysis of integral plasma membrane proteins. Anal Chem 76: 1817–1823.

Supporting information

Additional Supporting Information may be found in the online version of this article at the publisher’s web-site:

Fig. S1. Staining of the surface Gal/GalNAc lectin subunit in EhRabB8A gene-silenced (gs) cells. The EhRabB8A-gs and mock cells were fixed and stained with the anti-lectin heavy (A) and red (B). Phagocytic cup was stained with phalloidin-Alexa Fluor 488 (green). Bars, 10 μm.

Fig. S2. Sequence alignment (A) and percentage identity (B) of EhRabB8A, EhRab11B, Homo sapiens HsRab8A, HsRab10 and Saccharomyces cerevisiae Sec4p. Amino acid sequences were aligned with the CLUSTALW (ver 2.1). The GTP-binding consensus sequence alignment (A) and percentage identity (B) of EhRabB8A, EhRab11B, Homo sapiens HsRab8A, HsRab10 and Saccharomyces cerevisiae Sec4p. Amino acid sequences were aligned with the CLUSTALW (ver 2.1). The GTP-binding consensus region and switch I and II regions, which are involved in the interaction with effectors, are shown in the yellow box and red lines respectively (A). Amino acid percentage identity matrix was created by CLUSTALW (B).

Fig. S3. Immunoblot analysis using newly raised antibodies. The wild-type trophozoite was suspended with 1× SDS sample buffer supplemented with 0.5 mg/ml of E64, a cysteine protease inhibitor; the total cell lysate was then prepared by freeze–thawing. The lysate was applied to SDS-PAGE without boiling in order to avoid protein degradation, particularly for BiP. The following antibody dilutions were used: anti-Sec13 at 1:1000, anti-BiP at 1:1000 and anti-Rab11B at 1:1000.

Fig. S4. EhRabB8A did not localize to the lysosome and phagocytic cup. A. Lysosomes were stained with Lyso Tracker Red (red), and myc-EhRabB8A cells were subjected to immunofluorescence assay using anti-myc antibody (green). B. After 5 min of phagocytosis of erythrocytes, myc-EhRabB8A cells were fixed and subjected to immunofluorescence assay using anti-myc antibody (red). Phagocytic cup was stained with phalloidin-Alexa Fluor 488 (green). Bars, 10 μm.
report (Mi-ichi et al., 2009) and subjected to immunoblot using anti-Cpn60 (mitosome), anti-EhRab8A and anti-Sec13 antibodies respectively. The first Percoll fractions that are positive for Cpn60 were subjected to the second Percoll fractionation. Membrane-bound Sec13 was fractionated to J to L, and Cpn60 was detected from the L to M fractions. EhRab8A was co-fractionated with the Sec13-positive fraction (J and K).

Fig. S6. Subcellular localization of myc-EhRab8A wild type and mutants. The GTP-bound Q66L or GDP-bound S21N mutants were expressed under the regulation of the constitutive cysteine synthase promoter. Exogenous myc-EhRab8A was stained with the anti-myc 9E10 antibody. GTP-bound myc-EhRab8A Q66L was localized to the endoplasmic reticulum stained with the anti-BiP antibody, and the pattern was similar to that of the wild-type myc-EhRab8A. GDP-bound myc-EhRab8A S21N showed cytosolic staining.