BRIEF REPORT

Heavy subunit of cell surface Gal/GalNAc lectin (Hgl) undergoes degradation via endo-lysosomal compartments in *Entamoeba histolytica*

Kuldeep Verma[§] and Sunando Datta

Department of Biological Science, Indian Institute of Science Education and Research Bhopal, Bhauri, India

ABSTRACT

The human gut parasite *Entamoeba histolytica* uses a multifunctional virulence factor, Hgl, a cell surface transmembrane receptor subunit of Gal/GalNAc lectin that contributes to adhesion, invasion, cytotoxicity and immune response in the host. At present, the physiologic importance of Hgl receptor is mostly known for pathogenicity of *E. histolytica*. However, the molecular mechanisms of Hgl trafficking events and their association with the intracellular membrane transport machinery are largely unknown. We used biochemical and microscopy-based assays to understand the Hgl trafficking in the amoebic trophozoites. Our results suggest that the Hgl is constitutively degraded through delivery into amoebic lysosome-like compartments. Further, we also observed that the Hgl was significantly colocalized with amoebic Rab GTPases such as EhRab5, EhRab7A, and EhRab11B. While, we detected association of Hgl with all these Rab GTPases in early vacuolar compartments, only EhRab7A remains associated with Hgl till its transport to amoebic lysosome-like compartments.

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Introduction

Entamoeba histolytica is an intestinal protozoan parasite and the causative agent of invasive amebiasis. *E. histolytica* infect around 50 million people worldwide and approximate 100,000 dies annually.^{1,2} The highest estimated prevalence of the infections is mainly occurring in developing countries due to poor sanitation standard.

The D-galactose and N-acetyl-D-galactosamine (Gal/GalNAc) specific surface lectin of *E. histolytica*, has important roles in adhesion of the intestinal mucin and induction of adaptive immune response in human.³ The 260-kDa Gal/GalNAc lectin is a heterodimer of the transmembrane heavy subunit (Hgl, 170 kDa) and glycosylphosphatidylinositol (GPI)-anchored light subunit (Lgl, 35/31 kDa) glycoproteins linked by disulfide bonds.⁴ The Hgl subunit contains a carbohydrate recognition domain (CRD) that recognizes D-galactose and N-acetyl-D-galactosamine and important for adhesion and extracellular matrix degradation (ECM).^{5–8} The lectin complex binds to galactose, the N-acetyl-D-galactosamine and mucin glycoproteins on host cell surfaces and mediates both colonisation and contact-dependent cytotoxicity.^{9–11} In addition, this receptor is also involved in the capping and uroid formation in amoebic

trophozoites against the host immune responses.¹² Recent studies from our group has suggested a possible role of Hgl in the host ECM mediated actin dot formation in the amoeba⁸ which has implication in amoebic invasion. Cell surface localization of Hgl and its pivotal role in pathogenesis triggered initiation of few recent studies in which it has been investigated as a potential candidate for vaccine development.^{13,14}

Cell surface receptors play important role in host of biological processes. The surface population of the receptors is maintained by the two distinct cellular processes, transcriptional regulation and intracellular trafficking. Receptor population on the cell surface is maintained by opposing flux resulting from endocytosis and recycling.¹⁵ Several plasma membrane receptors from higher eukaryotes such as epidermal growth factor (EGF), integrins, transferrin, low-density lipoprotein (LDL) receptors are well characterized which undergoes lysosomal degradation or recycles back to plasma membrane depending on the extracellular cues.¹⁶

Rab GTPases are master regulators of vesicular trafficking.^{17,18} Earlier studies showed that the expression of either dominant negative¹⁹ or dominant active²⁰ mutant of amoebic RabA results in mis-localization of Hgl. In

CONTACT Sunando Datta sunando@iiserb.ac.in

[§]Present address: Regional Center for Biotechnology, NCR Biotech Science Cluster, Faridabad - 121 001, India

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recent studies from our group, we have shown that Hgl is localized to phagosomal membrane in association with EhRab7A during *E. coli* phagocytosis²¹ and EhRab35 during erythrophagocytosis.²²

The objective of the current study is to investigate the itinerary of intracellular vesicular trafficking of Hgl in *E. histolytica*. Furthermore, we also identified the endocytic Rab GTPases which associate with Hgl

Figure 1. (For figure legend, see page 3.)

along the endo-lysosomal route toward lysosomal degradation.

Results and discussion

In the previous studies, it has been shown that the Hgl localized to the plasma membrane and intracellular vacuolar compartments.^{20,23} In addition, Hgl was detected in late phagosomal fractions by proteomics-based study.²⁴ Here, we began with investigating the sub-cellular localization of Hgl using confocal microscopy on paraformaldehyde-fixed trophozoites. Our confocal microscopy based observation suggests that Hgl localized on the plasma membrane as well as on vacuoles (Fig. 1A). Earlier studies have established that amoeba harbors lysosome-like acidic compartments which facilitates the degradation of phagosomal and endosomal cargos.^{25,26} Here, we investigated whether Hgl is degraded in amoebic lysosome-like compartments similar to many other mammalian membrane proteins including β -integrins, transferrin, LDL and EGF receptor. Interestingly, the cytoplasmic domain of Hgl shares sequence similarity with $\beta 2$ and $\beta 7$ -integrins cytoplasmic tails, including amino acids that are implicated in the integrins mediated inside-out signaling.²⁷ LysoTracker-labeled amoebic trophozoites were processed for immunofluorescence using anti-Hgl antibody. A significant amount of colocalization ($r = 0.22 \pm 0.06$, $n = 100$ cells) was observed between Hgl and amoebic lysosome-like compartments (Fig. 1B) indicating a possibility of lysosomal degradation of the receptor. To biochemically explore this possibility the amoebic trophozoites were treated with cycloheximide, an inhibitor of protein synthesis²⁸ commonly used to investigate the degradation of cell surface receptors. The total amount of Hgl was analyzed by

immunoblot using anti-Hgl antibody at 0, 4 and 6 hrs post treatment with cycloheximide. As shown in Fig. 1C, the total amount of Hgl was found to reduce with time in the 100 $\mu\text{g/ml}$ cycloheximide treated trophozoites, suggesting that Hgl is being degraded over time. Neutralization of acidic compartments by lysosomotropic compound like NH_4Cl is a standard technique for inhibiting the degradation of the surface proteins by impairing the lysosome function.^{29,30} We used this method to further support our conclusion that intracellular degradation of Hgl is indeed mediated by lysosomal activity. Amoebic trophozoites, incubated in the presence of 20 mM NH_4Cl in combination with 100 $\mu\text{g/ml}$ cycloheximide for 0, 4 and 6 hrs. The treatment of NH_4Cl resulted in the reduction in degradation of Hgl at 4 and 6 hrs (Fig. 1C), suggesting that the lysosomal activity contributes to Hgl degradation. The lack of complete absence of degradation also suggests that the amoeba also uses lysosome independent degradation of Hgl.

Next, we developed an antibody uptake assay using monoclonal 3F4 Hgl (895-998 amino acid)^{31,32} antibody which recognizes the carbohydrate recognition domain of Hgl. Antibody uptake assay is one of the most powerful ways of studying the trafficking of the membrane receptors within cells. To avoid the internalization, trophozoites were incubated with 3F4 anti-Hgl antibody at 4°C for 30 min. After washing the unbound antibody, trophozoites were shifted to 37°C to initiate the internalization of the Hgl bound complex of the antibody. Trophozoites were fixed at different time points such as 15, 30, 60 and 120 min and processed for immunofluorescence (Fig. 1D). The internalized antibody was detected initially at the cell surface (15 min) and then in intracellular vacuoles (30 and 60 min). At a longer chase time (120 min) the

Figure 1. (see previous page) Hgl is constitutively degraded by lysosomes-like compartments in *E. histolytica* trophozoites. (A) Localization of Hgl on cell surface and intracellular vacuoles. Amoebic trophozoites were incubated on glass surface for 30 min at 37°C. The paraformaldehyde fixed trophozoites (non permeabilized, NP) were permeabilized (P) with 0.1% Triton X 100 and then immunostained with anti-Hgl antibody. Scale bar, 10 μm . (B) LysoTracker-labeled trophozoites were incubated on glass surface for 20 min at 37°C. The cells were fixed, and then immunostained with anti-Hgl antibody. The green arrowheads indicate Hgl and red arrowheads indicate LysoTracker compartments. The white arrowheads indicate colocalization of Hgl with LysoTracker-positive compartments. The zoomed panel show magnified views of the boxed areas. Scale bar, 10 μm . (C) Amoebic trophozoites were incubated with 100 $\mu\text{g/ml}$ cycloheximide (CHX) and CHX along with 20 mM NH_4Cl for 4 and 6 hrs. Cell lysates were analyzed by SDS-PAGE followed by immunoblotting with anti-Hgl (3F4 and 7F4) antibody and anti-EhCS1 (cytosolic control) antibody. Signal intensities for bands of Hgl and EhCS1 were quantified using the ImageJ program, and relative levels of Hgl were plotted against times. Data are normalized by considering the 0 hrs time point as a 100%. (D) Hgl (3F4) antibody uptake assay. Amoebic trophozoites were incubated with monoclonal anti-Hgl (50 $\mu\text{g/ml}$) antibody and then chase for 15, 30, 60 and 120 min at 37°C. After each time point trophozoites were fixed and immuno-stained with anti-mouse Alexa 488 conjugate and DAPI. The zoomed panel show magnified views of the boxed areas. Scale bar, 10 μm . (E) LysoTracker-labeled amoebic trophozoites were incubated with anti-Hgl (50 $\mu\text{g/ml}$) antibody and then chase for different time points at 37°C. After each time point trophozoites were fixed and process for immunofluorescence assay. The green arrowheads indicate Hgl and red arrowheads indicate LysoTracker compartments. The white arrowheads indicate colocalization of Hgl with LysoTracker-positive compartments. The zoomed panel show magnified views of the boxed areas. Scale bar, 10 μm . (F) Colocalization between Hgl and LysoTracker. Pearson's correlation coefficient (r) for colocalization between the Hgl and LysoTracker was quantified using the ImageJ-Fiji software. Data points shown in bar graph represent mean $\pm$ SDs. Significant differences are shown by * $P < 0.05$ and *** $P < 0.001$.

internalized antibody was accumulated as a patch in the trophozoites (Fig. 1D). Further, similar experiments were performed in the LysoTracker-labeled trophozoites (Fig. 1E). The intracellular localization of the antibody-Hgl complex was followed at different chase time using confocal microscopy. Our results suggest that the colocalization of Hgl with LysoTracker steadily increased with chase time. After 30 min incubation, only a subtle amount of Hgl co-localized with LysoTracker ($r = 0.06 \pm 0.02$, $n = 206$ cells) (Fig. 1F). At latter chase times, 60 min and 120 min significant colocalization was observed ($r = 0.12 \pm 0.03$, $n = 219$ cells and $r = 0.16 \pm 0.04$, $n = 212$ cells, respectively)

(Fig. 1F). Thus, our data suggests that amoebic trophozoites internalized the Hgl receptor and further delivers it to lysosome-like compartments. EGF receptor shows similar degradation pattern in mammalian cells and its intracellular trafficking has been investigated extensively in presence of different ligands. Upon the EGF binding, the receptor undergoes internalization with eventual degradation in lysosomes,³³ while amphiregulin binding triggers recycling of the internalized receptor to the cell surface.³⁴ Thus, depending on the nature of ligand EGF receptor undergoes either degradation or recycling which in turn regulates its response to a given extracellular cue. Lysosomal

Figure 2. Colocalization between Hgl and Rab GTPases. (A) Amoebic trophozoites were incubated on glass surface for 30 min at 37°C. The cells were fixed, and then immunostained with anti-Hgl and anti-EhRab5 or EhRab7A or EhRab11B antibodies. The green arrowheads indicate Hgl and red arrowheads indicate Rab GTPases (EhRab5, EhRab7A and EhRab11B). The white arrowheads indicate colocalization of Hgl with respective Rab GTPases. The zoomed panel show magnified views of the boxed areas. Scale bar, 10 μ m. (B) Colocalization between Hgl and amoebic Rab GTPases (EhRab5, EhRab7A, EhRab11B). Colocalization was quantified using the ImageJ-Fiji software to obtain Pearson's correlation coefficient (r). Data points shown in bar graph represent mean $\pm$ SDs. Number of cells used for each amoebic Rab and Hgl colocalization [(EhRab5, $n = 198$ cells), (EhRab7A, $n = 205$ cells) and (EhRab11B, $n = 209$ cells)]. Significant differences are shown by * $P < 0.05$ and ** $P < 0.01$.

degradation along with recycling is also observed for $\beta 1$, $\beta 2$ and $\beta 3$ - integrin receptors.³⁵⁻³⁷ The constitutive degradation of Hgl also could be a mechanism of immune evasion adapted by the parasite to survive

and invade the host. Albeit, our study does not rule out the possibility that the anti-Hgl antibody could influence the degradation of Hgl in amoebic lysosome-like compartments. In mammalian cells some of the

Figure 3. (For figure legend, see page 6.)

monoclonal antibodies against EGF receptor³⁸ and transferrin receptor³⁹ are known to induce lysosomal degradation of these receptors.

We further wanted to investigate the intracellular itinerary of Hgl. Rab GTPases along with their respective effectors play an essential role in intracellular membrane trafficking of cell surface receptors.¹⁸ Here we used a set of Rab GTPase such as EhRab5, EhRab7A and EhRab11B which are known to regulate early, late or secretory pathways, in *E. histolytica*.^{25,40} While EhRab5 and EhRab7A are localized on early endosomal or phagosomal compartments, EhRab7A continues being associated with late endosomes/phagosomes.^{21,25,41,42} Thus both these Rabs are part of amoebic endo-lysosomal compartments. In contrast, EhRab11B was found to be associated with the secretion of cysteine proteases⁴⁰ perhaps suggesting its role in secretory or recycling trafficking of the membrane receptor. Here, we investigated the colocalization of these amoebic Rab GTPase with Hgl. Amoebic trophozoites were incubated on the glass surface for 30 min at 37 °C and processed for immunofluorescence using anti-Rab and anti-Hgl antibodies (Fig. 2A). A quantitative colocalization analysis revealed that Hgl is significantly colocalized with EhRab5 ($r = 0.57 \pm 0.08$, $n = 198$ cells), EhRab7A ($r = 0.39 \pm 0.08$, $n = 205$ cells) and EhRab11B ($r = 0.52 \pm 0.13$, $n = 209$ cells) (Fig. 2B) suggesting its strong association with the endocytic and exocytic or recycling compartments.

To further investigate the itinerary of Hgl through EhRab5 and EhRab7A positive compartments to amoebic lysosome-like compartments, we performed antibody uptake assay. Amoebic trophozoites were incubated with Hgl antibody at 4°C for 30 min. Trophozoites were then incubated at 37°C to initiate internalization of the antibody bound cell surface Hgl. The cells were fixed at 30, 60 and 120 min and processed for immunofluorescence using anti-EhRab5 and anti EhRab7A antibody to identify the endo-lysosomal compartments. Confocal images were acquired and used for quantitative analysis to

measure colocalization of Hgl and Rab compartments (Fig. 3A). Our results suggest that at 30 and 60 min of the chase time Hgl is mostly accumulated in EhRab5 or EhRab7A positive compartments. At a later time point, only EhRab7A remain associated with the Hgl containing compartments (Fig. 3B). Earlier studies have shown that EhRab7A is involved in the transport of amoebapore to RBC phagosomes⁴¹ as well as *E. coli* clearances in the amoebic lysosome-like compartments.²¹ Therefore, we asked whether some of the EhRab7A positive Hgl compartments are acidified, representing amoebic lysosome-like compartments. LysoTracker-labeled trophozoites were used for antibody uptake assay. Further, the internalization of antibody was chased for 120 min at 37°C and processed for immunofluorescence using anti-EhRab7A antibody. We found significant triple colocalization between Hgl, EhRab7A and LysoTracker suggesting that Hgl continues to be associated with EhRab7A till it's delivery into amoebic lysosome-like compartments (Fig. 3C).

Here, we demonstrated that the Hgl is internalized and trafficked via EhRab5, EhRab7A, and EhRab11B positive compartments to amoebic lysosome-like compartments for degradation. The association of Hgl with these Rab proteins might imply for their involvement in its trafficking but further experiments would be necessary to establish their role. Recently, the role of EhRab5 has been shown in biogenesis of endocytic vacuoles in the amoeba which are involved in transport of iron binding protein transferrin.²⁵ Similarly, EhRab7A has been shown to be associated with *E. coli* containing phagosome and plays important role in degradation of the bacteria in lysosome-like compartments.²¹ In a separate study, we also detected EhRab35 to be associated with RBC phagosomes and important for acidification of amoebic lysosome-like compartments.²² Hgl was found on the EhRab35 positive RBC phagosomes. Thus, a possible role of EhRab35 in lysosomal degradation of Hgl could not be ruled out. In the current study, we have observed significant colocalization of Hgl with

Figure 3. (see previous page) Hgl association with Rab GTPases in endo-lysosomal compartments. (A) Amoebic trophozoites were incubated with anti-Hgl (50 µg/ml) antibody and then chase for different time points at 37°C. After each time point trophozoites were fixed and process for immunofluorescence assay using EhRab5 and EhRab7A antibodies. The *green* arrowheads indicate Hgl and *red* arrowheads indicate Rab compartments. The *white* arrowheads indicate colocalization of Hgl with Rab-positive compartments. The *cyan* arrowheads indicate Rab compartment which is not positive for Hgl. Scale bar, 10 µm. (B) Amoebic trophozoites were incubated with anti-Hgl (50 µg/ml) antibody and then chase for different time points at 37°C. After each chase time, cells were fixed and process for immunofluorescence assay using EhRab5 and EhRab7A antibodies. Confocal images were acquired and used for quantitative association between Hgl and Rab GTPases. Twenty trophozoites were used for quantification in each condition and plotted. Data points shown in bar graph represent mean ± SDs. Number of both Hgl and Rab positive compartments/Total number of Hgl vacuoles or vesicles x 100. Scale bars, 10 µm (C) LysoTracker- labeled amoebic trophozoites were incubated with anti-Hgl (50 µg/ml) antibody and then chase for 120 min at 37°C. After trophozoites were fixed and process for immunofluorescence assay. The *blue* arrowheads indicate EhRab7A, *green* arrowheads indicate Hgl and *red* arrowheads indicate LysoTracker compartments. The *white* arrowheads indicate the triple positive compartments for EhRab7A, Hgl and LysoTracker. Scale bar, 10 µm.

EhRab11B. In mammalian cells, Rab11 family members regulate both exocytosis⁴³ and recycling of endocytic cargoes.⁴⁴ The cellular functions of amoebic Rab11 members have been investigated in earlier studies.^{40,45,46} While, EhRab11B, due to its involvement in secretion of proteases was postulated to localize on recycling endosomes,⁴⁰ EhRab11A was shown to be important for the amoebic encystation.^{45,46} The functions of the other Rab11 family members in the amoeba are yet to be revealed. From the current study, we do not have any evidence of recycling of Hgl from the endocytic compartments to cell surface.

Taken together, this study provides the first evidence of constitutive degradation of Hgl in lysosome-like compartments in the parasite and their association with endocytic and exocytic Rab GTPases. It would be interesting to detect whether the degradation of Hgl depends on any extracellular cues which will provide insight into regulation of cell surface activity of this virulence factor under different pathophysiological contexts. In addition the current study opened up new avenues for identification of amoebic Rab GTPases which are specifically involved in the regulation of Hgl degradation in *E. histolytica*.

Materials and methods

Culture of *Entamoeba histolytica* trophozoites

E. histolytica strain HM1:IMSS trophozoites were grown axenically in BI-S-33 medium supplemented with 15% (v/v) heat-inactivated adult bovine serum (Cat. RM9981, Himedia, India) and 100 U of penicillin/ml and 100 µg streptomycin sulfate/ml (Life Technologies, CA, USA), at 35.5 °C as described previously.⁴⁷

LysoTracker staining

For LysoTracker labeling, amoebic trophozoites were incubated in the BI-S-33 medium containing LysoTrackerTM Red DND-99 (Cat. L-7528, Life Technologies, USA) at a final concentration of 2 µM for 2 hour at 35.5 °C.

Antibody-uptake assay and immunofluorescence assay

To analyze the localization of anti-Hgl (3F4) monoclonal antibody in the antibody-uptake experiments. Logarithmic grown wild-type trophozoites were harvested from glass tube and washed with ice-cold 1X PBS and incubated with 50 µg/ml anti-Hgl (3F4) in DMEM Gluta-MAX (Cat. 10566016, Life Technologies, CA, USA)

supplemented with 0.5% BSA and 20 mM HEPS at 4 °C for 30 min on a platform rocker (150 rpm). After, trophozoites were incubated with 10 volumes with DMEM medium and centrifuge at 900 g for 3 min at 4 °C. The trophozoite and antibody complex was suspended in BI-S-33 medium and transferred into 8-well chamber slides (Cat. 30108, SPL Life Sciences, Singapore) at 37 °C for different time points. After each time points, trophozoites were fixed with 4% paraformaldehyde and permeabilized with 0.1% Triton X-100 in 1XPBS. After blocking (5% fetal bovine serum in 1XPBS) trophozoites were incubated for 1 hr in 1:100 dilution of primary antibodies rabbit polyclonal anti-EhRab7A, anti-EhRab5, anti-EhRab11B and mouse monoclonal anti-Hgl (3F4) at room temperature. After 3 washes, trophozoites were co-incubated with Alexa-conjugated (Life Technologies, CA, USA) secondary antibodies (1:500 dilutions) and DAPI (4',6-diamidino-2-phenylindole) for 1hr at room temperature. After 3 washes with 1XPBS solution, coverslips were mounted on the glass slide using Mowiol. Slides were examined using a LSM-780 laser scanning confocal microscope (Carl Zeiss, GmbH, Jena, Germany) with a 63 ×/1.4 NA oil immersion objective lens. Immunofluorescence signals were captured in individual en face (*xy* axes) planes throughout the cellular *z*-axis at 0.4 µm intervals.

Colocalization analysis

To quantify colocalization, an entire image was selected as a region of interest, and analysis was carried using ImageJ-Fiji Colocalization Threshold (developed by Tony Collins,⁴⁸ McMaster Biophotonics Facility, McMaster University, Hamilton, ON, Canada; available at <http://fiji.sc/Fiji>). Pearson's correlation coefficient(*r*) was calculated between the 2 fluorescent signals. Values represent the means and standard deviations (SD) from each image.

Western blotting

E. histolytica trophozoites were solubilized with lysis buffer containing 50 mM Tris-Cl pH 7.5, 150 mM NaCl, 1 mM PMSF, 1 mM DTT and 1% NP-40 in the presence of protease inhibitor cocktail (Cat. P8340, Sigma Aldrich, USA). Proteins were resolved at 100 V on 8% or 15% polyacrylamide gels under reducing conditions. Proteins were transferred electrophoretically to nitrocellulose membranes, and blots were incubated with 5% BSA (Bovine Serum Albumin) blocking buffer for an hour at room temperature and probed with mouse monoclonal anti-Hgl (3F4 and 7F4) (1:35) and rabbit polyclonal anti-EhCS1 [cytosolic control (1:1000)]. Further, it was

washed and incubated with HRP-conjugated anti-rabbit (1:10000) and anti-mouse (1:6000), and finally developed by enhanced chemiluminescence.

Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

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Author contributions

KV and SD conceived and designed the experiments and wrote the paper. KV performed the experiments. All authors analyzed the results and approved the final version of the manuscript.

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