Autophagy during proliferation and encystation in the protozoan parasite Entamoeba invadens

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Abbreviations: Eh, Entamoeba histolytica; Ei, Entamoeba invadens; Atg8, Autophagy-related gene 8; PE, phosphatidylethanolamine; PAS, pre-autophagosomal structure.

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Abstract

Autophagy is one of the three systems responsible for degradation of cytosolic proteins and organelles. Autophagy has been implicated in the stress response to starvation, antigen cross presentation, defense against invading bacteria and viruses, differentiation, and development. Yeast Atg8 and its mammalian ortholog LC3 play an essential role in autophagy. The intestinal protozoan parasite *Entamoeba histolytica* and a related reptilian species *E. invadens* possess the Atg8 conjugation system, consisting of Atg8, Atg4, Atg3, and Atg7, but lack the Atg5-Atg12 conjugation system. Immunofluorescence imaging revealed that polymorphic Atg8-associated structures emerged in the logarithmic growth phase, and decreased in the stationary phase, as well as in the early phase of encystation in *E. invadens*. Immunoblot analysis showed that increase of phosphatidylethanolamine-conjugated membrane-associated Atg8 was also accompanied with the emergence of Atg8-associated structures during the proliferation and differentiation above mentioned. Specific inhibitors of class I and III phosphatidylinositol 3 kinases simultaneously inhibited both growth of trophozoites and autophagy, and also both encystation and autophagy in *E. invadens*. These results suggest that core machinery for autophagy is conserved and plays an important role during proliferation and differentiation in *Entamoeba*.

Keywords: Amoebiasis, *Entamoeba histolytica*, encystation, autophagy, Atg8.
Introduction

Amoebiasis is a diarrheal disease caused by the protozoan parasite *Entamoeba histolytica*, and affects approximately fifty million inhabitants of endemic areas resulting in an estimated 40,000-110,000 deaths annually (61). Its transmission occurs by ingesting the infective cysts in contaminated food or water (33). Although encystation is an essential fundamental process required for transmission of the disease, little is known on the biochemical and cell biological changes as well as the regulation of gene expression that occur during this process except for some cases (5, 7, 10-12, 47, 57). In a reptilian *Entamoeba* species *E. invadens*, 18 genes including ubiquitin 48, gene 122 (47), chitinase 1 and 2 (7, 57), two isoforms of chitin synthases (chs-1 and chs-2) (5), cyst-specific lectins called Jacob (7) and Jessie (5, 57) were differentially expressed at different stages of encystation. However, various fundamental events that occur during encystation, which allow drastic changes in cellular compositions and organelle structures remain largely uncharacterized except for the involvement of proteasomes (12). Recently, a transcriptome of *E. histolytica* clinical isolates has been shown, which identified some novel *E. invadens* stage-specific genes (10).

Autophagy is a cellular process highly conserved in eukaryotes, which permits the degradation of long-lived proteins and damaged or unnecessary organelles (24). Autophagy has been implicated in various biological processes including the stress response to carbon and nitrogen starvation, antigen cross presentation, defense against invading bacteria, viruses and other intracellular pathogens, differentiation, and development (15, 25, 28, 30, 38, 49, 51). Autophagosome formation is initiated by the emergence of an isolation membrane in the cytosol, also called as the “pre-autophagosomal structure (PAS)” (36, 50, 58). PAS elongates and expands to form the autophagosome membranes (19, 25, 36) that enclose cytosolic components and organelles including mitochondria and endosomes (14, 18, 45). Autophagosomes subsequently fuse to lysosomes leading to the degradation of sequestered...
organelles and materials (15, 25). In yeast, 16 autophagy-related (Atg) genes that encode
components essential for autophagy were identified (21). Autophagosome formation is
regulated by two related ubiquitin-like (UBL) systems in both yeast and mammals (25,54).
The first UBL system allows conjugation of Atg12 to Atg5. After the Atg12-Atg5 conjugation,
they subsequently form a complex with Atg16 (22) and this complex acts upstream of the
second conjugation system. In the latter system, where Atg8 is a major anchoring molecule,
Atg4 protease (54) exposes the terminal glycine of Atg8 (36). Atg8 is subsequently activated
by the E1 enzyme Atg7 (54) and transferred to Atg3, a E2-like enzyme, which conjugates
Atg8 to phosphatidylethanolamine (PE) (17). Atg4 also participates in the Atg8 deconjugation,
which is essential for the fusion of autophagosomes with lysosomes/vacuoles (2, 4, 20, 54, 62).
Among the components involved in these conjugation systems, Atg8 has been considered to
be an authentic marker for autophagosomes because lipid-anchored Atg8 remains attached to
the inner membrane of autophagosomes until it is degraded by lysosomal hydrolases after the
fusion with lysosomes (36).

The biological importance of autophagy has been demonstrated both in vitro and in
vivo in various organisms (9, 16, 23, 29, 44, 63). In Caenorhabditis elegans, inhibition of
Atg1, Atg6, Atg7, Atg8, and Atg10 by RNA interference resulted in a defect in the
morphogenesis of Dauer, a dormant form produced between the L2 and L3 larval stages under
conditions of high population density, reduced nutrients, or increased temperature, where
development is ceased to sustain survival for an extended period (34). In the social ameba
Dictyostelium discoideum, Atg8 plays a role in survival and spore formation. During
starvation, D. discoideum develops a multicellular form that functions like spore reservoir. A
mutant lacking Atg5 or Atg7 was unable to produce viable, mature spores and the
autophagosome formation was abolished under starvation (41). A Leishmania major vps4-
mutant, which showed a defect in endosomal sorting and the fusion of Atg8-autophagosomes
with lysosomes, failed to differentiate from a diving procyclic promastigote into the infective
metacyclic form (4).

In this study, we show by a genome-wide survey that *E. histolytica* and related
reptilian species *E. dispar* and *E. invadens* possess genes involved in the Atg8 conjugation,
but not those of the Atg5-Atg12 conjugation pathway. Furthermore, we demonstrate, by
immunoblot and immunofluorescence assays, that autophagy occurs in two independent
phases of its life cycle, during the logarithmic growth phase and the early phase of encystation,
in *E. invadens*.

**Materials and Methods**

**Bacteria, chemical, and reagents.** *E. coli* DH5α and BL21(DE3) strains were
purchased from Life Technologies (Tokyo, Japan) and Invitrogen (Tokyo, Japan),
respectively. All chemicals of analytical grade were purchased from Sigma-Aldrich (Tokyo,
Japan) or Wako (Tokyo, Japan) unless otherwise stated.

**E. invadens culture and encystation.** Trophozoites of *E. invadens* IP-1 strain were
cultured axenically in BI-S-33 medium at 26°C. To induce encystation, two-week-old *E.
invadens* cultures were passaged to the 47% LG medium lacking glucose (47) at
approximately 6 x 10^5 cells/ml. Amoebae were collected at various time points and the
formation of cysts was assessed by virtue of the resistance to 0.05% sarkosyl using 0.22%
trypan blue to selectively stain dead cells. Cysts were also verified by cyst wall staining by
incubating amoebae with Calcofluor white (Fluorescent brightener 28, Sigma-Aldrich) at
room temperature.

**Genome-wide survey of genes involved in autophagy in Entamoeba.** *S. cerevisiae*
Atg proteins were obtained from NCBI non-redundant protein database (http://www.ncbi.nlm.nih.gov/) and used as queries to search for orthologs in the *E. histolytica*, *E. dispar*, and *E. invadens* genome database (http://www.tigr.org/tdb/e2k1/eha1; http://www.sanger.ac.uk/Projects/E_histolytica/; http://www.sanger.ac.uk/Projects/E_dispar/; and http://www.sanger.ac.uk/Projects/E_invadens). Possible orthologs were further analyzed with the blastp algorithm (http://www.ncbi.nlm.nih.gov/blast/) against the non-redundant database at NCBI to find the closest homologues in other organisms.

**Production of recombinant *E. histolytica* Atg8 (EhAtg8) and antiserum against EhAtg8.** Standard techniques were used for routine DNA manipulation, subcloning, and plasmid construction as previously described (46). The protein coding region of *EhAtg8a* (XP_649165) was amplified using oligonucleotide primers (5’-CATCCCGGGATGGAATCACAACCAAAACTT-3’ and 5’-AGACTCGAGTTAATTTCCAAAGACAGATTC-3’) and *E. histolytica* cDNA library (39) by PCR. Parameters used for PCR are: an initial step of denaturation at 94°C for 2 min, followed by 30 cycles of denaturation at 94°C for 15 sec, annealing at 55°C for 30 sec, and extension at 65°C for 1 min. A final step at 95°C for 9 sec, 60°C for 9 sec, and 95°C for 9 sec was used to remove primer dimers. A 408-bp PCR fragment was cloned in the SmaI-XhoI site of the pGEX-6P-2 expression vector (GE Healthcare Bioscience, Tokyo, Japan) to produce pGST-EhAtg8. pGST-EhAtg8 plasmid was introduced into *E. coli* BL21(DE3) strain. The expression of GST-EhAtg8 recombinant protein was induced and cell lysate was produced according to instructions by the manufacturer. The GST tag at the amino terminus of the recombinant GST-EhAtg8 was cleaved by Precision protease (GE Healthcare Bioscience) and removed by GSTrap (GE Healthcare Bioscience). The recombinant EhAtg8 protein was further purified by Mono Q anion exchange chromatography on AKTA Explorer (GE Healthcare Bioscience). Rabbit antiserum against EhAtg8 was commercially produced.
Cell Fractionation. Amoebae were harvested, washed with phosphate-buffered saline containing 2% glucose, and resuspended in a homogenization buffer (50 mM Tris pH 7.5, 250 mM sucrose, 50 mM NaCl, and 1.34 mM trans-epoxysuccinyl-L-leucylamido-(4-guanidino butane). The amoebae were homogenized with 80-300 strokes (depending on the percentage of cysts) in a glass homogenizer and centrifuged at 400 g for 5 min at 4°C to remove unbroken cells. The supernatant was further centrifuged at 100,000 g for 1 h to separate a high-speed pellet and supernatant, which were subjected to immunoblot analysis.

Immunoblot analysis. To differentiate unmodified and PE-conjugated Atg8, SDS-polyacrylamide gel electrophoresis was conducted using 13.5% separating gel containing 6M urea as previously described (20). Approximately 5 µg of lysates or fractions were separated by denaturing SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes. After the membranes were blocked with 5% skim milk, they were incubated with anti-EhAtg8 or anti-EhNifS (1) antibody (1/1000 dilution) for 1 h. After washing, the membranes were incubated with anti-rabbit IgG alkaline phosphatase-conjugated (Jackson Lab, Main, USA) or horse radish peroxidase-conjugated antibody (Amersham Bioscience, Piscataway, NJ, USA) for 1 h. Proteins were visualized by AP Conjugate Substrate Kit (BIO-RAD, Hercules, CA, USA) or by chemiluminescence with Immobilon Western (Millipore Corporation, Billerica, MA, USA). The intensity of bands visualized by chemiluminescence was detected on Lumi-Imager F1 (Roche Applied Science, Tokyo, Japan) with the LumiAnalyst software (Roche Applied Science) and further analyzed with ImageJ.

Indirect immunofluorescence assay. Trophozoites and cysts were fixed in 3.7% paraformaldehyde in phosphate-buffered saline for 10 minutes at room temperature, washed, and permeabilized with 0.2% saponin for 10 minutes. Then, cells were incubated with anti-
EhAtg8 antibody (1/1000 dilution) for 1 h at room temperature, and subsequently incubated with anti-rabbit IgG Alexa Flour 488 antibody (Invitrogen) for 1 h at room temperature. Finally, cells were washed, mounted on a slide glass, examined under a confocal laser scanning microscope (Carl Zeiss LSM 510 META, Thornwood, NY, USA). Images were further analyzed with LSM 510 software.

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Results and Discussion

One of the two ubiquitin-like systems is conserved in E. histolytica. In yeast and mammals, the biogenesis of autophagosomes is regulated by two ubiquitin-like (UBL) systems (25, 54). The E. histolytica genome [HM1:IMSS (8)] apparently encodes only one of the two UBL systems (Table 1). We identified genes encoding two Atg8 (designated as EhAtg8a and EhAtg8b), four Atg4 (designated as EhAtg4a-d), one Atg7, and one Atg3. The two EhAtg8 proteins showed 96% mutual identity. EhAtg8a and EhAtg8b showed 31-33, 25-27, and 28-30% identity to bee Apis mellifera Atg8a, yeast Atg8, and GABA receptor-like 2 (Gabarap1) from amphioxus Branchiostoma belcheri tsingtauense, which was shown to be expressed during the early embryo and larvae development (26).

EhAtg4a to EhAtg4d showed 22-26% identity against yeast or mammalian Atg4. Atg4 belongs to a new class of cysteine proteases (20), which differ in function and structure from other cysteine proteases such as papain, HAUSP, UCH-L3, and Ulp1 families (52). EhAtg4s lack a prodomain typically present in most of amoebic CPs. In addition, the active-site cysteines that are well conserved among cathepsin L-like CPs, e.g., CP1, CP2, and CP5, are not aligned with these Atg4s (data not shown). These EhAtg4s were previously reported as “autophagins 1-4”, which are members of the C54-cysteine endopeptidase family (6). In yeast only one isoform of Atg4 has been reported (53, 55), while Homo sapiens apparently
possesses four Atg4 homologs (52). The putative Atg3 and Atg7 homologue (EhAtg3 and EhAtg7) showed 31-47% and 34-38% identities to yeast or mammalian counterparts, respectively. On the other hand, E. histolytica apparently lacks genes encoding Atg5, Atg10, Atg12, and Atg16 (Table 1).

The presence of the Atg8 conjugation system and the concomitant absence of the Atg5-Atg12 conjugation pathway were also reported for both the free-living social amoeba Dictyostelium (41) and the parasitic protist Leishmania (60). These data may indicate that the Atg8 conjugation system is evolutionarily older than the Atg5-Atg12 conjugation system, and that the former represents minimal machinery required for autophagy. As shown below, the Atg8 conjugation system is apparently sufficient for the autophagosome biogenesis at least in these protists (4, 41).

Conservation of the Atg8 conjugation pathway in related Entamoeba species, E. dispar and E. invadens. E. dispar is the commensal non-pathogenic species for mammals, while E. invadens is the invasive reptilian species and a primary model of encystation.

Although E. dispar and E. invadens genomes have not been completed, we identified two Atg8 (AANV01003233 and AANV01014404), one Atg4 (AANV01002875), two Atg7 (AANV01000460 and AANV01009354), and one Atg3 (AANV01000567) in the E. dispar database, while only one Atg8 (AANV01005912, nt 44-433), one Atg4 (AANV01010411, nt 5-1166, likely containing an intron, the carboxyl terminus incomplete) and one Atg3 (AANW01009890, nt 424-1226 in a complementary strand) were found in the E. invadens genome database (data not shown). We were unable to identify an Atg7 ortholog in the E. invadens genome, likely due to incomplete sequencing of the genome. Comparison of Atg8 from E. invadens (EiAtg8) and E. dispar (EdAtg8) against EhAtg8s showed that EiAtg8 is 99 or 95% identical to EhAtg8a or EhAtg8b, while EdAtg8 is 98 or 96% identical to EhAtg8a or EhAtg8b, respectively. The high degree of conservation in E. invadens is remarkable.
considering that this species has shown to be largely divergent from *E. histolytica* and *E. dispar*, as indicated by the significant differences in the codon usage and the G-C content between *E. invadens* and *E. histolytica / E. dispar* (40).

**Features of *Entamoeba* Atg8.** Amino acid comparisons with Clustal W (Fig. 1) highlighted a number of unique features of *Entamoeba* Atg8. Phe77 and Phe79 (numbered for *ScAtg8*), implicated for the interaction with Atg4 in yeast (2) are not conserved in Atg8 from *Entamoeba* species, while these residues are well conserved in *Leishmania*, *Arabidopsis*, and mammals. Neither are other important residues involved in the autophagy activity in yeast (Tyr 49 and Leu 50) (2) conserved in *Entamoeba* Atg8. A number of other residues totally conserved in Atg8 from other organisms are not conserved in *Entamoeba*; these residues are distributed throughout the entire protein (overlined in Fig. 1).

All *Entamoeba* Atg8s share a unique 16-amino acid insertion (e.g., a.a. 72-88 of *EhAtg8a*: YIETDGETPISTVSVK). Based on the crystal structure of Atg8 from yeast, this region is located 1-5 residues upstream of the Atg4 recognition site (Phe-X-Phe), which is absent in *Entamoeba* Atg8 and thus predicted to be exposed on the surface of the molecule and possibly interacts with other proteins. We also examined the nucleotide sequence of this region of all *Entamoeba* Atg8s, and excluded the possibility that this region corresponds to an intron by reverse transcriptase PCR of cDNA (data not shown). *EhAtg8b* has an extra 5-amino acid amino-terminal extension (MDPTF), which is also present in *EdAtg8*, but missing in *EiAtg8*. Interestingly, the overall identity of *EdAtg8* and *EiAtg8* to *EhAtg8a* or *EhAtg8b* is not consistent with the fact that *EiAtg8* lacks the 5-amino acid amino-terminal extension present in *EhAtg8b*, while *EdAtg8* possesses it. It is unlikely that the lack of this amino-terminal extension in *EiAtg8* is due to incomplete genome database because the orf of *EiAtg8* is not located at the end of the contig AANW01005912 (nt 44-433 of the total length of 1166 nt).
**Immunoblot analysis of Atg8 in *E. invadens*.** In order to verify if autophagy occurs in *Entamoeba*, and, if it does, to identify growth phases and stages where autophagy plays a role, immunoblot analysis of *E. invadens* trophozoites cultured in a regular proliferation medium with anti-EhAtg8 antibody was conducted (Fig. 2). Two bands of approximately molecular mass of 15.0 and 14.5 kDa were observed (Fig. 2A). Dramatic changes in both the total amount of Atg8 and the proportion of the two forms were found during two weeks of culture. The intensity of both 15.0- and 14.5-kDa Atg8 bands increased in the early and mid logarithmic growth phase (days 4-12), and peaked at day 9, while it decreased at days 2 and 15. The ratio of the intensity of the 15.0-kDa band to that of the 14.5-kDa band was 2.4, 1.4, 1.1, or 2.2 at day 4, 7, 9, or 12, respectively, based on chemiluminescence measurement of these bands (data not shown). These data correlate well with the results of immunofluorescence study (Fig. 3, see below).

Cell fractionation followed by immunoblot assay (Fig. 2B) showed that the 15.0-kDa Atg8 was fractionated almost exclusively to the soluble, likely cytosolic, fraction, while the 14.5-kDa Atg8 was found in the 100,000 x g pellet fraction. Based on a number of other studies (17, 20), we concluded that the top band corresponds to the cytosolic non-conjugated Atg8, while the bottom band to the membrane-associated PE-conjugated Atg8.

**Immunofluorescence imaging of Atg8 in proliferating *E. invadens* trophozoites.** Immunofluorescence analysis revealed remarkable changes in the Atg8-associated autophagosome-like structures in *E. invadens* trophozoites in both logarithmic and stationary growth phases. Microscopic images of cultures at representative time points, one and two weeks, under low magnification showed homogeneous Atg8-staining in each population, but remarkable differences in intensity were observed between one and two week-old cultures (Fig. 3, A, B). In order to further investigate the observed growth phase-dependent synthesis and degradation of autophagosomes, we quantified the number of Atg8-associated structures,
examined under high magnification, during exponential to stationary growth phases (Fig. 3, D-F). The average number of Atg8-associated autophagosome-like structures (see below for morphological categories) peaked at 9 days in the BI-S-33 proliferation medium. Although the number of the Atg8-associated structures was >3 per cell at day 2, the structures were mainly dot-like or tiny vesicles (data not shown), which is consistent with the repression of Atg8 demonstrated by immunoblot analysis.

Expression and membrane recruitment of Atg8 change during encystation in E. invadens. Autophagy has recently been implicated in differentiation during nutrient deprivation in organisms such as C. elegans, D. discoideum, and Leishmania (4, 34, 41, 60). Although it has been recently demonstrated that encystation-specific genes could be identified in E. histolytica clinical isolates (10), in vitro encystation of both E. histolytica clinical isolates and laboratory strains has not been accomplished. To further investigate if autophagy is induced during encystation, we exploited encystation of E. invadens with two week-old cultures as an inoculum into the glucose-lacking 47% LG medium. Under the given conditions we accomplished up to 80-93% encystation within 96 h, based on sarkosyl resistance (Fig. 4A). We also verified that kinetics of the increment of sarkosyl-resistant amoebae and that of Calcofluor-stained amoebae were almost indistinguishable (data not shown). Although encystation is usually induced in trophozoites using 2-3 day-old (12) or 7 day-old culture in the logarithmic growth phase (32), we used two week-old cultures as an inoculum. As described above, the amount of Atg8 protein and the number of membrane-associated Atg8-positive structures were significantly lower in the two week-old cultures than 2-7 day-old cultures, and thus the kinetics of Atg8 synthesis and the formation of the Atg8-positive structures during encystation could be ideally monitored by using two week-old cultures.

Amoebae were harvested at various time points and Atg8 was first examined by
immunoblots and quantitated as above (Fig. 4, B and C). The amount of unmodified Atg8
reduced as early as 2 h after transferring the amoebae to the encystation medium.
Concomitantly, the amount of PE-modified Atg8 increased and peaked at 24-48 h. The total
amount of Atg8, estimated by chemiluminescence assay, changed during encystation. The
ratio of PE-conjugated Atg8 to unmodified Atg8 also changed significantly. The ratio of
Atg8-PE to unmodified Atg8 also increased in a course of encystation (e.g., 0.09 or 1.26 at 6
or 96h, respectively). These data are similar to the observation on the formation of
autophagosomes under starvation conditions in other organisms (19). Interestingly, at 24 h
post-induction of encystation, the production of the PE-modified Atg8 reached a plateau,
while only 7% of trophozoites differentiated into cysts as evaluated with the detergent
resistance (Fig. 4A) and Calcofluor staining (data not shown). Thus, the emergence of PE-
conjugated Atg8 precedes encystation. To verify autophagy really occurs in encysting cells,
we examined the presence of Atg8-associated structures in detergent-resistant cysts after
Sarkosyl treatment. Although we were unable to simultaneously examine cyst markers and
Atg8 due to technical problems (i.e., Calcofluor can not be used in IFA), these data are
consistent with the premise that cluster formation increases as encystation precedes.

**Immunofluorescence assay reveals dramatic changes in the autophagosome-like
structures during encystation.** Immunofluorescence imaging of encysting *E. invadens
cultivated in the 47% LG medium showed that both the percentage of amoebae containing
structures and the number of the structures per amoeba increased during encystation (Fig. 5).
Representative stacks of Atg8 structures at 24h post-induction of encysting *E. invadens* are
shown in Fig. 6. We morphologically categorized the Atg8-associated structures into several
groups: “dots” (containing no luminal region), “vesicles/vacuoles” of <1, 1-2, 2-4, or >4µm in
diameter (containing a luminal region surrounded by Atg8-positive structures), “others”
(including linear or thread-like structures), “clusters” (clustered structures composed of
multiple dots/vesicles/vacuoles and other structures) (Fig 5, A and C). Atg8-positive dots and
other structures, including linear and diffuse/amorphous structures, were observed in almost
all cells in the population. As early as 2 h post-induction of encystation, both the percentages
of cells containing the Atg8-positive dot-like and other structures as well as the numbers of
these structures per cell started to increase, peaked at 24 h, and then gradually decreased at
24-96 h. Both the number and size of the vesicular/vacuolar structures increased at 4-24 h;
e.g., at 24 h, approximately 65% of amoebae contained large Atg8-positive vesicles/vacuoles
of 2 to 4 µm. The Atg8-positive clusters emerged only after 24 h. Concomitant with the
emergence of the cluster structures, the percentage of cysts started to increase, suggesting the
cluster formation may coincide with the initiation of morphological and biochemical
encystation.

The dot structures observed in this study were similar to that typically referred as PAS
(Fig. 5C, filled arrowhead), which corresponds to a perivacuolar site containing lipids and
early autophagy proteins such Atg8/LC3 (27). PAS undergoes elongation originating a double
membrane called isolation membrane (36, 58). It was reported that in yeast, the diameter of
the autophagosomes ranges from 0.3 to 0.9 µm (58), while it usually ranges up to 1.5 µm in
mammals (e.g., embryonic stem cells, embryonic fibroblasts, hepatocytes, and pancreatic
acinar cells) (35). Therefore, the size of the Atg8-associated autophagosome-like structure in
Entamoeba, shown in the present study, are unprecedented, except for the 5 to 10-µm
autophagosomes containing group A Streptococcus and Mycobacterium tuberculosis (13, 37).

Under non-starvation conditions we predominantly found 1-2-µm Atg8-positive
vesicles/vacuoles in proliferating trophozoites, suggesting that vesicles/vacuoles of this size
represent autophagosomes constitutively formed under normal conditions. This is similar to
what was reported for mammalian cells where autophagy was constitutively active or
suppressed in response to specific hormones (58).
PI 3-kinase inhibitors abolish proliferation, encystation, and the formation of autophagosomes in *E. invadens*. Proliferation of trophozoites was severely affected by wortmannin, a well known fungal PI 3-kinase inhibitor, which was shown to inhibit autophagy in yeast, mammals, and *Leishmania* (4, 27). The growth of trophozoites cultivated in the BI-S-33 medium containing 1 µM wortmannin for one week was impaired by > 90% (data not shown). Immunofluorescence analysis showed that the average number of the Atg8-associated structures decreased by 25% in trophozoites treated with the inhibitor (data not shown).

We next examined effects of inhibition of PI 3-kinase by wortmannin or 3-methyladenine during encystation. Wortmannin at >100 nM completely inhibited encystation as measured with the Sarkosyl resistance at 48 h (Fig. 7B). Concomitantly, 100nM of wortmannin also inhibited rounding and agglutination of parasites, which was normally observed in the late logarithmic growth phase (Fig. 7A). These data are consistent with the previous findings (31). Immunofluorescence assay showed that the formation of Atg8-associated structures was abolished by wortmannin (Fig. 7C-E). Both the percentage of positive cells and the number of Atg8-associated clusters and vesicles/vacuoles, of particularly of 2-4 and > 4µm, per cell significantly decreased by the wortmannin treatment (Fig. 7, B and C). Although the percentage of cells containing dots did not change, the average number of these punctate structures, which presumable correspond to PAS, was reduced by 50% (p<0.05). The simultaneous dose-dependent inhibition of encystation and the autophagosome formation is consistent with the involvement of PI3K in the biogenesis, most likely fusion and/or aggregation, of autophagosomes in *Entamoeba* and that autophagy plays an important role in encystation. Both encystation and the formation of Atg8-associated structures were also simultaneously inhibited by 3-methyladenine although the effective concentrations were >100 fold higher than those of wortmannin (data not shown). It was
previously shown that wortmannin inhibits PI 3-kinases with an IC$_{50}$ of ~10 nM, while 3-methyladenine is less potent than wortmannin (its IC$_{50}$ is ~$10^6$-fold higher than wortmannin (27). Wortmannin and 3-methyladenine inhibit the class III PI 3-kinases including Vps34 (42), which forms the PI 3-kinase complex I (58), an essential component of PAS (36) by competition of ATP binding (27). E. histolytica apparently possesses at least three Vps34 (XP_656932), Vps15 (XP_652375), and Apg6/Beciclin 1 (XP_656050) of the four components necessary to form the PI 3-kinase complex, while the remaining component, Apg14, is absent in its genome. However, since proliferation, autophagy, and survival were shown to be regulated by Tor kinase, class I and III PI3K (25, 27, 48, 56), the possibility cannot be excluded that the defect of proliferation or encystation is not causally connected, but simply coincides with the inhibition of autophagy.

**Significance of autophagy in Entamoeba.** The presence of autophagosome-like structures in proliferating trophozoites of both *E. invadens* and *E. histolytica* (data not shown, described elsewhere) suggest a house-keeping role of autophagy in this group of protists. The remarkable increase in the formation of autophagosomes in the mid to late logarithmic growth phase and the repression in the stationary phase are consistent with the hypothesis that autophagy likely play a role in proliferation, but not in survival during starvation. Involvement of autophagy during proliferation was also suggested for mouse T cell (43). In addition to a role in proliferation, the dramatic formation of autophagosomes during encystation of *E. invadens* strongly suggest autophagy is involved in differentiation. The fact that the emergence of the Atg8-associated structures precedes morphological and biochemical changes (7, 10, 12, 47) during encystation, strongly indicates that autophagy is prerequisite for and plays an important role in encystation. It is also important to note that most of differentially expressed genes previously identified (47) were up-regulated between 22-24 h post-encystation induction, which coincides with the peak of autophagosome formation.
Rapid degradation and recycling of cellular components via autophagy may be advantageous during encystation, which requires dramatic and swift reorganization of cellular structures and organelles.

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FIGURE LEGENDS

Figure 1. Alignment of Atg8 protein sequences from three *Entamoeba* species and other organisms.

Clustal W (http://www.ch.embnet.org/software/ClustalW.html) and BoxShade 3.21 (http://www.ch.embnet.org/software/BOX_form.html) were used to produce alignment. Conserved amino acid residues are shown in black and conserved substitutions are shown in gray. Gaps are shown in dashes. An asterisk under the alignment indicates the consensus glycine that is conjugated to PE. Residues implicated for binding to Atg4 and the autophagy activity were depicted with triangles and squares under the alignment, respectively. *Entamoeba*-specific residues are overlined. Sequences used in the alignment are: *Ed*Atg8 (*E. dispar*, accession no. AANV01003233), *Ei*Atg8 (*E. invadens*, AANW01005912, nt 44-433), *Eh*Atg8a (*E. histolytica*, XP_649165), *Eh*Atg8b (*E. histolytica*, XP_649940), *Dd*Atg8 (*Dictyostelium discoideum*, XP_637841), *Lm*Atg8.1 (*Leishmania major*, CAJ07266), *Sc*Atg8 (*Saccharomyces cerevisiae*, P38182), *Dm*Atg8a (*Drosophila melanogaster*, NP_727447), *Am*Atg8 (*Apis mellifera*, XP_001120069), *At*Atg8e (*Arabidopsis thaliana*, NP_182042), **GBRL2_Bb** (GABARAP like-2 from *Branchiostoma belcheri*, AAO45172), **GBRL2_Mm** (GABARAP like-2 from *Mus musculus*, BAB22217).

Figure 2. Immunoblot analysis of Atg8 in *E. invadens*.

(A) Immunoblot analysis of Atg8 in *E. invadens* trophozoites cultured in a normal proliferation medium. *E. invadens* trophozoites were cultivated in BI-S medium at indicated times (2-15 days) and whole lysates were electrophoresed by SDS-polyacrylamide gel electrophoresis, blotted, and reacted with anti-Atg8 (top panel) or a control antibody.
against *EhNifS* (1) (bottom panel). A representative blot of three independent experiments was shown. (B) Cell fractionation of Atg8. A whole lysate produced by mechanical homogenization of trophozoites, harvested at 1 week after initiation of the culture at cell density of ~5x10^4 cell/ml was separated to the supernatant and pellet fraction of 100,000 x g centrifugation, and subjected to immunoblot analysis as above. H, a whole homogenate; S or P, a supernatant and a pellet of 100,000 x g centrifugation, respectively.

**Figure 3. Immunofluorescence imaging of autophagy in proliferating trophozoites of *E. invadens*.**

(A-C) Immunofluorescence images of Atg8 in proliferating *E. invadens*. Maximum projection images of 8-10 *E. invadens* trophozoites of 1- (A) and 2-week-old (B) cultures reacted with anti-*EhAtg8* antibody under low magnification are shown. One-week old culture was also reacted with preimmune serum (C). (D-E) Confocal images under high magnification of a representative slice of a single typical trophozoite harvested at 1 (D) or 2 weeks (E). (F)

Growth kinetics of *E. invadens* trophozoites and autophagosome formation in the normal proliferation medium. Cell density was measured in triplicate and its mean and S.D. are shown in diamonds and a solid line. Trophozoites in proliferation were assayed by immunofluorescence as previously described; the average number of Atg8-positive structures per positive cell was counted and shown in bars. Approximately 30-40 cells were examined at each time point. Pair-wise comparisons between each time point, with statistical significance (p values < 0.001, 0.01, or 0.05), are also indicated.

**Figure 4. Immunoblot analysis of Atg8 in *E. invadens* during encystation.**

(A) Kinetics of encystation. The percentage of the amoebae resistant to 0.05% Sarkosyl during encystation is shown. (B) *E. invadens* trophozoites were harvested at indicated times,
lysed, fractionated as in Fig. 2B, and subjected to immunoblot analysis by using anti-\(Eh\)Atg8 (top panel) or anti-\(Eh\)NifS antibody (bottom panel), followed by chemiluminescence detection.

H, whole homogenate; S, supernatant; P, pellet of 100,000 x g centrifugation. (C) Densitometric measurement of immunoblots developed by chemiluminescence. Only representative quantitation of three immunoblots, which showed similar kinetics, is shown.

Figure 5. Kinetics of Atg8-positive autophagosome-like structures during encystation by immunofluorescence assays. (A) The percentage of amoebae containing categorized Atg8-positive structures during encystation is shown. Categories are: cytosolic; dot-like; vesicles/vacuoles with the diameter of <1.0 µm, 1.0-2.0 µm, 2.0-4.0 µm and, >4.0 µm; other structures; and clusters. (B) The average number of Atg8-structures, as categorized above, per positive amoeba. Approximately 30-40 cells were examined at each time point, and means and S.D. are shown. Pair-wise comparisons between each time point, with statistical significance (p values < 0.001, 0.01, or 0.05), are also indicated. Representative results of three independent experiments are shown in (A) and (B). (C) Subcellular localization of Atg8 during encystation. Representative images of maximum projection of approximately 20 slices taken at 1-µm intervals on z-axis at each time point are shown. Filled arrowheads, open arrowheads, arrows, or asterisks indicate Atg8-positive dot-like structures, clusters, vesicles/vacuoles, or other structures, respectively. Bars = 5 µm.

Figure 6. Confocal images of Z stacks of an encysting \(E. invadens\) trophozoite. \(E. invadens\) trophozoites were incubated in 47% LG medium for 24 h and analyzed by immunofluorescence assay with anti-\(Eh\)Atg8 antibody and Alexa 488-conjugated anti-rabbit IgG antibody. Sixteen slices of a representative trophozoite were captured at 1-µm intervals on z-axis and shown in an order of the bottom to the top. Filled arrowheads, open arrowheads,
arrows, or asterisks indicate representative Atg8-positive dot-like structures, clusters, vesicles/vacuoles, or other structures, respectively.

Figure 7. Inhibition of encystation and the formation of autophagosomes by wortmannin in *E. invadens*.

(A) Micrographs of amoebae at 72 h post-induction of encystation with or without wortmannin. *E. invadens* trophozoites were incubated in 47% LG and supplemented with DMSO (control) or 100nM wortmannin. (B) The percentage of cysts after 2, 24, or 72 h of incubation in the encystation medium with various concentrations of wortmannin. Results of a representative set of three independent experiments are shown. (C) Immunofluorescence images of *Ei*Atg8-positive structures in cells untreated or treated with 1 or 10 µm wortmannin in the encystation medium for 2 days. Representative images of maximum projection of approximately 20 slices taken at 1-µm intervals on z-axis are shown. (D) The percentage of amoebae containing categorized Atg8-positive structures during encystation in the presence of 1 or 10 µm wortmannin is shown. (E) The average number of Atg8-positive structures per positive amoeba in the absence or presence of 1 or 10 µM wortmannin. Representative results of three independent experiments are shown.
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$^a$NCBI database
$^b$E. histolytica genome database at TIGR
Figure 2

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Figure 4

A

Percentage of cysts vs. Time (hours)

B

Western blot analysis at different time points:
- Atg8
- Atg8-PE
- NifS

C

Intensity (arbitrary unit) vs. Time (hours)

Ratio of Atg8-PE/Atg8
Figure 5

A

Percentage of cells with Atg8-positive structures

Time (hours)

B

Number of Atg8-structures per positive cell

Time (hours)

† p < 0.05,  * p < 0.01, †* p < 0.001

C

0 min  2 h  4 h  6 h  24 h  48 h  72 h  96 h

Downloaded from http://iai.asm.org/ on November 12, 2017 by guest
Figure 7

A

control

1 μM

B

Percentage of cysts

Time (hours)

- DMSO
- 10 μM
- 1 μM
- 100 nM
- 1 nM
- 0.01 nM
- 0.001 nM

C

control

1 μM

D

Percentage of cells with Atg8-structures

vesicles/vacuoles

- DMSO
- 1 μM wortmannin
- 10 μM wortmannin

E

Number of Atg8-structures per positive cell

vesicles/vacuoles

- DMSO
- 1 μM wortmannin
- 10 μM wortmannin