Article Addendum

Endoplasmic reticulum continuity in the protozoan parasite Entamoeba histolytica

Evolutionary implications and a cautionary note

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Abbreviations: ER, endoplasmic reticulum; GFP, green fluorescent protein; FLIP, fluorescence loss in photobleaching; HSP-70, heat shock protein 70; Gal/GalNAc, galactose/N-acetyl-D-galactosamine

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Entamoeba histolytica has been described as an early branching eukaryotic parasite based on the lack of organelles such as mitochondria and peroxisomes, and on morphologic studies that concluded it possesses a vesicular endoplasmic reticulum (ER) and Golgi complex. However, a recent study from our laboratory showed that the E. histolytica ER is continuous by using an ER-targeted green fluorescent protein fusion protein and photobleaching experiments. We proposed that the vesicular ER seen earlier was likely an artifact of fixation. We now report data using an alternative fixation protocol that preserves the continuous ER morphology. These data confirm that the vesicular ER reported earlier was indeed a fixation artifact; furthermore, since we observed the same ER structure when staining for the native antigen HSP-70 in wild-type amebae, the data provide direct evidence that the continuous ER morphology we reported is correct. This work has important implications for cell biologists studying E. histolytica virulence, emphasizes the frequent need to reassess assumptions based on published data, and provides additional evidence that E. histolytica actually diverged relatively late in evolution and that many of its unusual features are likely due to loss of features during adaptation to its ecological niche.

Entamoeba histolytica is the intestinal protozoan parasite that causes amebiasis, a disease characterized by dysentery that may affect 50 million people annually.1,2 The role of secreted proteins in virulence and their effect on host cells has been studied extensively.3,4,5 E. histolytica’s secretory pathway is yet to be understood. Entamoeba histolytica trophozoites lack mitochondria and peroxisomes.5 Furthermore, based on several morphologic studies that used transmission electron microscopy and confocal microscopy to examine fixed amebae, typical Golgi and endoplasmic reticulum (ER) structures have been believed to be absent from E. histolytica trophozoites.6-9 Based on these morphologic features, some consider E. histolytica to be a “living fossil” that diverged from other eukaryotes prior to development of these organelles.10 However, this belief runs contrary to accumulating data that suggest E. histolytica diverged from the main eukaryotic lineage relatively recently, including studies demonstrating remnants of mitochondrial DNA in E. histolytica (suggesting secondary loss of mitochondria rather than divergence prior to acquisition), results of small subunit ribosomal RNA sequencing, and identification of several features of a more typical secretory pathway including secretion of glycosylated proteins, presence of a homologue to the ER retention receptor Erd2, and the results of the E. histolytica genome projects.5,11-14

In a recent paper, we demonstrated that E. histolytica in fact has a continuous ER network like that of other eukaryotic cells.15 Using E. histolytica trophozoites expressing a FLAG-epitope tagged green fluorescent protein (GFP) that was targeted to the ER by fusion to the ER retention sequence KDEL at the carboxyl terminus and the amino terminal signal sequence of a galactose/N-acetyl-D-galactosamine (Gal/GalNAc) specific E. histolytica surface lectin, we were able to observe the ER morphology in living amebic trophozoites for the first time. The ER-targeted GFP fusion protein was distributed in what appeared to be a continuous network reminiscent of the ER in Saccharomyces cerevisiae. The GFP-KDEL protein was excluded from pseudopodia, suggesting presence within a membrane bound compartment, and three-way junctions characteristic of the ER in other eukaryotes were readily visible (Fig. 1 and ref. 15). To test the continuity of this network experimentally, we used the method of fluorescence loss in photobleaching (FLIP). The live trophozoites were overlayed with soft agar to restrict parasite movement and a portion of the cell was bleached by a high beam laser. GFP fluorescence diminished rapidly from the entire cell while neighboring control cells were minimally bleached, indicating that...
the ER-targeted GFP fusion was freely diffusible within a single, continuous compartment. We concluded that the vesicular ER observed earlier was likely a result of fixing artifacts and, contrary to published data, that *E. histolytica* possesses a continuous ER.

One concern with our approach was that the reticular network observed might have resulted from overexpression of the GFP-KDEL fusion protein, a possibility we were unable to exclude completely. For example, this might be possible if protein overexpression induces so-called tubular transport intermediates (TTIs) in *E. histolytica*, which are linear expansions of the vesicular transport apparatus that have been observed in mammalian cells following protein overexpression.

We have sought to examine localization of native ER proteins to exclude this possibility. Since using fixed cells for immunofluorescent staining of native ER proteins is unavoidable, we first needed a fixation method that would preserve the ER phenotype seen in living trophozoites. Figure 1 shows new data using an altered fixation protocol that preserves the continuous ER morphology. *Entamoeba histolytica* trophozoites expressing the FLAG-tagged GFP-KDEL protein were fixed with either 3% or 4% paraformaldehyde for 20 minutes at room temperature, permeabilized with 0.2% Triton X-100, and labeled with a monoclonal anti-FLAG mouse antibody and an anti-mouse IgG Alexa 488 conjugated goat antibody. As previously described, the ER-targeted GFP protein appeared to be distributed in vesicles when fixed with 3% paraformaldehyde; simply increasing the paraformaldehyde concentration to 4% preserved the continuous ER phenotype seen in living trophozoites (Fig. 1A).

Using the new fixation protocol, co-staining of the FLAG-GFP-KDEL protein with either the representative ER protein HSP-70 or with the Gal/GalNAc specific lectin, a type I integral membrane protein present on the cell surface and within the secretory pathway, demonstrated excellent co-localization of these native proteins with GFP-KDEL in the ER compartment (Fig. 1B and C, respectively). Interestingly, HSP-70 was typically more abundant in the peripheral ER, while the GFP-KDEL fusion protein was distributed more homogenously, hinting at the likelihood of specialized regions within the *E. histolytica* ER (Fig. 1B and data not shown).

To further exclude the possibility that the continuous ER network could be induced by protein overexpression, we next used this fixation protocol to examine the distribution of HSP-70 in wild-type *E. histolytica* trophozoites. Again, a reticular network was observed, indicating that this phenotype was not an artifact of protein overexpression (Fig. 1D).

By providing direct evidence that the vesicular *E. histolytica* ER reported in earlier studies was a result of fixation artifacts and demonstrating that the continuous ER phenotype is observed in cells fixed by an alternate protocol and stained for native proteins, these data confirm and extend our previous report which concluded that the ER-targeted GFP fusion was freely diffusible within a single, continuous compartment. We concluded that the vesicular ER observed earlier was likely a result of fixing artifacts and, contrary to published data, that *E. histolytica* possesses a continuous ER.

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This observation is also important to cell biologists studying *E. histolytica*. Others and we have identified endoplasmic reticulum proteins in purified phagosomes while other investigators have found no ER proteins in the *E. histolytica* phagosome. Given this and conflicting reports regarding ER-dependent macrophage phagocytosis, we want to determine if *E. histolytica* uses ER-dependent phagocytosis under some circumstances. Using this alternative fixation protocol, the role of the ER in *E. histolytica* pathogenesis and phagocytosis can now be studied. Finally, these data stress the frequent need to reconsider assumptions based on published studies that by necessity were dependent on older technologies, and should
serve as a cautionary note for cell biologists by emphasizing the importance of using the best available methods.

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