NOTES

Evidence of a Continuous Endoplasmic Reticulum in the Protozoan Parasite *Entamoeba histolytica*[▽][†]

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Entamoeba histolytica, the cause of amebiasis, is believed to have no continuous endoplasmic reticulum (ER), with ER functions occurring in vesicles. Here, using an ER-targeted green fluorescent protein fusion protein and fluorescence loss in photobleaching, we have unambiguously demonstrated the presence of a continuous ER compartment in living *E. histolytica* trophozoites.

Entamoeba histolytica, the intestinal protozoan that causes invasive amebiasis, is an important source of morbidity and mortality in developing countries (1). Beyond its medical importance, *E. histolytica* has several unusual features that are of interest to evolutionary biologists. It is a structurally simple eukaryote, lacking mitochondria and peroxisomes and having no recognizable Golgi complex or rough endoplasmic reticulum (ER) (6, 11, 16). Some have hypothesized that *E. histolytica* diverged from other eukaryotic lineages prior to the development of these structures (2). However, there is strong evidence for the secondary loss of mitochondria in *E. histolytica*, and small-subunit rRNA sequence analyses suggest that it diverged more recently than several lineages with typical eukaryotic features (3, 17). Proof of N-linked protein glycosylation in *E. histolytica*, the identification of membrane proteins with recognizable N-terminal signal sequences and of functional C-terminal ER retention peptides, and data from the genome project indicate that the basic vesicle-trafficking machinery of other eukaryotes is conserved in *E. histolytica* (8, 10, 12). Based on transmission electron microscopy and confocal microscopy studies of fixed cells, the current belief is that the *E. histolytica* ER is comprised of vesicles of various sizes (8, 13).

We used green fluorescent protein (GFP) fusions to study the ER structure in living *E. histolytica* trophozoites for the first time. The GFP fusion proteins used included an untargeted GFP (predicted to localize to the cytosol) and GFP fused with an N-terminal signal sequence from the Gal/GalNAc-specific *E. histolytica* adherence lectin followed by a FLAG epitope and a C-terminal ER retention peptide, KDEL (FLAG-GFP-KDEL) (predicted to localize to the ER) (Fig. 1A). DNA
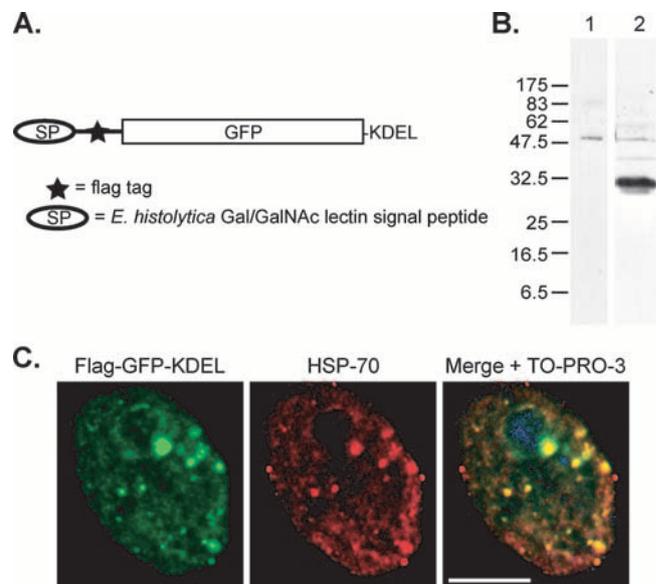

FIG. 1. Expression and visualization of an ER-targeted GFP fusion protein in fixed *E. histolytica* trophozoites. (A) The ER-targeted GFP construct. GFP was cloned in frame with an N-terminal signal sequence, the FLAG epitope tag, and the C-terminal ER retention peptide KDEL. (B) Immunoblot showing the stable expression of the ER-targeted GFP fusion in *E. histolytica* trophozoites. Whole amebic lysates from wild-type (lane 1) and transfected (lane 2) parasites were probed with an anti-FLAG monoclonal antibody and anti-IgG-horseradish peroxidase conjugate. Bound antibody complexes were detected by enhanced chemiluminescence. (C) Immunofluorescent confocal microscopy demonstrating the colocalization of the ER-targeted GFP fusion with the representative ER protein BiP in fixed amebic trophozoites. The vesicular appearance of the *E. histolytica* ER compartment is typical of the appearance previously reported using fixed cells. *Entamoeba histolytica* trophozoites on glass coverslips were fixed with paraformaldehyde, permeabilized, and stained with an anti-FLAG monoclonal mouse IgG antibody and an anti-BiP rabbit polyclonal serum followed by anti-mouse IgG-Alexa 488 (green) and anti-rabbit IgG-Alexa 568 (red) conjugates. The nucleus was stained with TO-PRO-3 and pseudocolored blue. Slides were examined with a confocal microscope. Original magnification, ×1,200. Scale bar, 10 μm.

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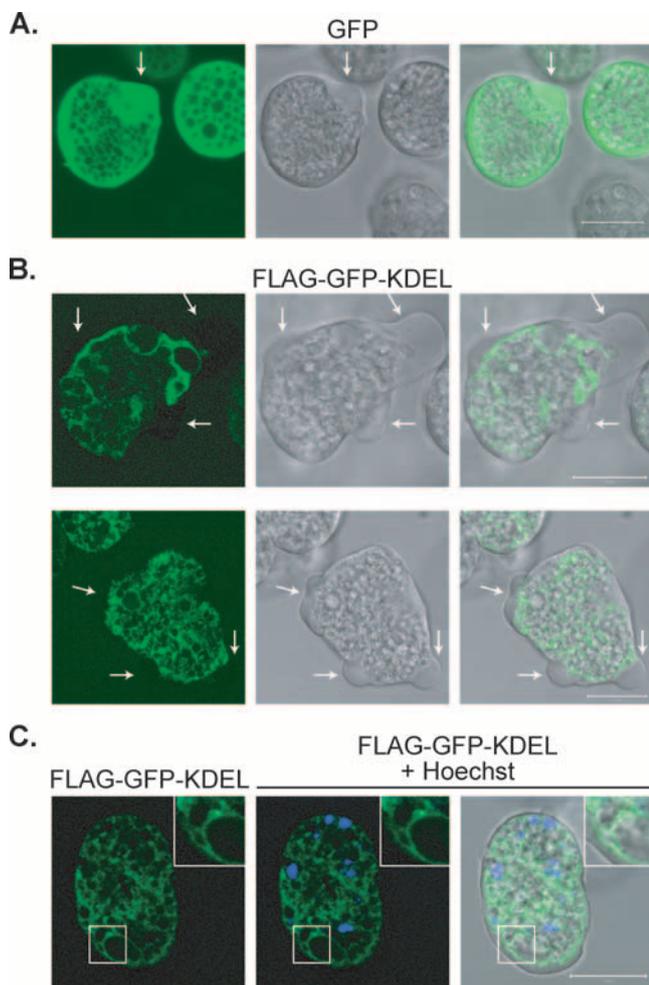
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FIG. 2. Visualization of untargeted GFP and the ER-targeted GFP fusion protein in living *E. histolytica* trophozoites. Parasites stably expressing each protein were allowed to adhere to glass-bottomed culture dishes and then were coated with a thin layer of agar to restrict their movement. The localization of each protein was examined using a confocal microscope. (A) Untargeted GFP was present diffusely in the cytosol, including within pseudopodia (arrow). (B) The ER-targeted GFP fusion protein localized to a widely distributed reticular network. The absence of the fluorescent fusion protein from pseudopodia (arrows) suggested that it was restricted to a membrane-enclosed compartment. (C) Nuclear costaining and polygonal reticulum. The nuclei in living cells were stained with Hoechst and were pseudocolored blue. Multiple nuclei were seen typically, and three-way junctions characteristic of the polygonal endoplasmic reticulum were readily visible (shown in the blown-up inset) (see panel B; also see Fig. 3 as well as Movie S1 in the supplemental material). Original magnification, ×1,260. Scale bars, 20 μm.
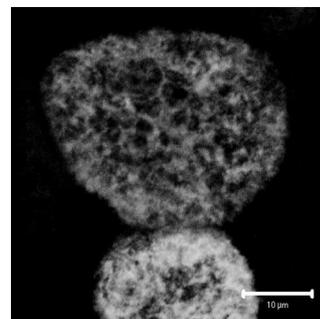

FIG. 3. Three-dimensional projection showing the location of the FLAG-GFP-KDEL protein in a living *E. histolytica* trophozoite. Parasites expressing the ER-targeted GFP fusion protein were allowed to adhere to glass-bottomed culture dishes and were covered with a thin layer of agar, and confocal z-sections were obtained. The three-dimensional projection shown was created using the Zeiss LSM software package (version 4.2). The ER-targeted fusion protein again was localized to a widely distributed reticular network (see Movie S1 in the supplemental material). Original magnification, ×1,953. A 10-μm scale bar is shown.

encoding both constructs was cloned by PCR into the *E. histolytica* expression vector pGIR235, which utilizes a Gal/GalNAc lectin promoter sequence to drive constitutive protein expression and carries a neomycin resistance cassette (15). The expression plasmids were introduced into *Entamoeba histolytica* strain HM-1:IMSS trophozoites using Lipofectamine. Stable transfectants were selected with 6 μg/ml G418, and the G418 dose was doubled weekly to a final concentration of 24 μg/ml prior to the use of the transfected cell lines in experiments. Figure 1B shows an immunoblot performed by probing whole wild-type or FLAG-GFP-KDEL-expressing *E. histolytica* lysate with an anti-FLAG mouse monoclonal antibody (M2 clone; Stratagene, La Jolla, CA) followed by an anti-mouse immunoglobulin G (IgG)-horseradish peroxidase conjugate. Bound antibody complexes were detected by enhanced chemiluminescence. The presence of a specific band of the predicted molecular weight in the FLAG-GFP-KDEL-transfected parasites confirmed the expression of the recombinant protein.

To determine if the FLAG-GFP-KDEL protein was properly localized within the ER compartment, we compared its localization to that of the representative ER chaperone BiP (HSP70) in fixed *E. histolytica* trophozoites by using immunofluorescent confocal microscopy (Fig. 1C). FLAG-GFP-KDEL-expressing trophozoites adherent to glass coverslips were fixed with 3% paraformaldehyde, permeabilized with 0.1% Triton X-100, and stained with the anti-FLAG mouse monoclonal antibody and an anti-BiP rabbit polyclonal serum (a gift from T. Nozaki, Gunma University, Japan) followed by goat anti-mouse IgG Alexa 488 and goat anti-rabbit IgG Alexa 568 antibodies (Invitrogen, Carlsbad, CA). The nucleus was visualized using the far-red fluorescent DNA binding dye TO-PRO-3 (Invitrogen). Slides were examined using a Bio-Rad MRC 1024ES confocal microscope. As has been reported previously for fixed *E. histolytica* trophozoites, the ER chaperone BiP was present in vesicles of various sizes, with increased abundance surrounding the nucleus (8). The FLAG-GFP-KDEL fusion protein showed excellent colocalization with BiP. No staining occurred either with a combination of the rabbit anti-BiP serum and the anti-mouse IgG Alexa 488 conjugate or with the mouse anti-FLAG antibody and the anti-rabbit IgG Alexa 568 conjugate (staining controls) (data not shown). We concluded that the GFP fusion protein was properly targeted to the ER compartment and that the FLAG-GFP-KDEL-expressing parasites would be useful for examining the ER in living *E. histolytica* trophozoites.

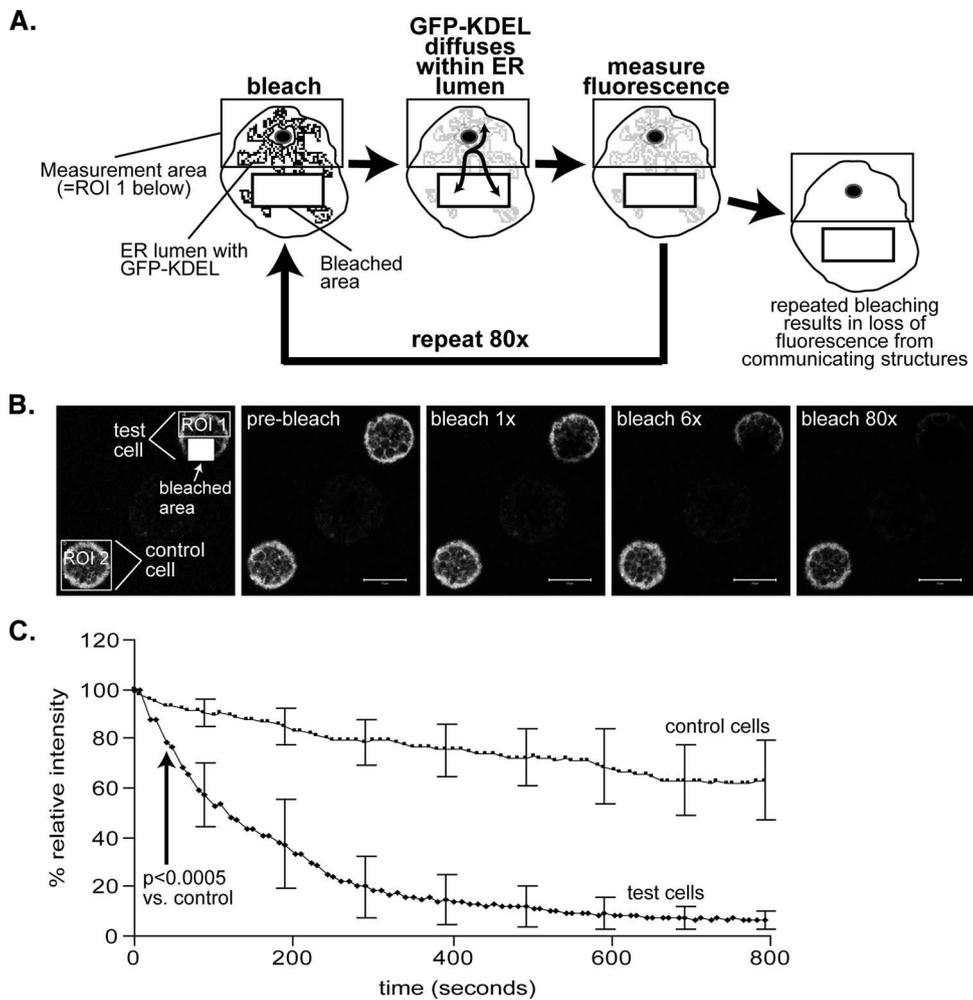We next compared the localization of the FLAG-GFP-

FIG. 4. Photobleaching experiment to assess ER continuity in *E. histolytica* trophozoites. To prevent excessive cell movement, parasites adherent to glass-bottomed culture dishes were coated with agar. (A) Experimental strategy for examining ER continuity using FLIP. The ER-targeted GFP fusion was photobleached repeatedly within a region of each test cell. The relative fluorescence intensity of a region within the same cell and within a neighboring, unbleached control cell (not depicted) was measured after each bleaching cycle. Since unbleached proteins diffused into the bleached region and were bleached during subsequent cycles, the entire ER compartment in the test cell was selectively photobleached. (B) Representative images taken during the execution of the photobleaching protocol. Shown, from left to right, are the experimental setup showing the bleached region and areas used, prebleach images, and images acquired following 1, 6, and 80 bleaching cycles. ROI 1 is the region of fluorescence intensity measured for the test cell. ROI 2 is the region of intensity measured for the control cell. Original magnification, ×945. Scale bars, 20 μm. (C) Time course showing the change in relative fluorescence within the bleached and control cells during repeated photobleaching. The initial values for the relative intensity were set to 100%. Values shown are the means and standard deviations of the relative fluorescence intensity at each time point ($n = 9$; $P < 0.0005$ compared to values for the control for time points at and beyond that indicated by the arrow).

KDEL protein to the localization of untargeted GFP in living parasites. Trophozoites adherent to glass-bottomed culture dishes (MatTek, Ashland, MA) were covered with a thin layer of Luria-Bertani (LB) top agar to limit parasite movement and were examined using a Zeiss 510 META confocal microscope. In some cases, the nuclei were stained with the cell-permeable DNA dye Hoechst 33258 (Polysciences, Warrington, PA). Untargeted GFP was present diffusely within the cytosol, including within pseudopodia (Fig. 2A). We were surprised to find the ER-targeted GFP fusion within a broadly distributed and apparently continuous reticular network that had a prominent peripheral component lying underneath the plasma membrane (Fig. 2B and C). In contrast to untargeted GFP, the FLAG-GFP-KDEL fusion protein was excluded from pseudopodia,

suggesting its retention within a membrane-enclosed compartment (Fig. 2B). As reported previously, trophozoites with multiple nuclei were common (7). The FLAG-GFP-KDEL protein typically was found in close proximity to the nuclei; furthermore, three-way junctions characteristic of the ER were readily observed (Fig. 2C).

These putative ER structures were highly reminiscent of the ER described for *Saccharomyces cerevisiae* (9, 14, 21). The overexpression of ER-targeted fusion proteins in mammalian cells has been shown to induce so-called tubular transport intermediates, the appearance of which correlates with the degree of protein expression, suggesting that they result from the saturation of the vesicle transport machinery (18). The FLAG-GFP-KDEL protein appeared to be aberrantly targeted

in a small minority of very brightly fluorescent trophozoites (data not shown), so we cannot exclude the possibility that structures analogous to tubular transport intermediates are induced in *E. histolytica*. However, the reticular structures shown in Fig. 2 were unlikely to be an artifact of protein overexpression, since, unlike tubular transport intermediates, their presence did not correlate with fluorescence intensity. To characterize this presumptive ER structure further, z-sectioning was performed using a ×63, 1.4-numerical-aperture objective and step sizes of 0.4 μm to achieve an optical thickness of 0.8 μm. This enabled the reconstruction of three-dimensional projections, which further suggested that the observed reticular network was continuous (Fig. 3) (also see Movie S1 in the supplemental material).

We used the method of fluorescence loss in photobleaching (FLIP) to test the hypothesis that *E. histolytica* has a single, continuous ER compartment. This method has been used previously to verify ER continuity in other cell types (4, 5, 19, 20). As depicted in Fig. 4A, one region of the cell is repeatedly photobleached, and the fluorescence intensity is measured in the remainder of the cell. If the GFP-tagged protein diffuses freely within a single compartment, then the fluorescence is rapidly lost from the entire cell. A nearby cell is included in the field to control for fluorescence loss due to repeated imaging. The FLAG-GFP-KDEL-expressing trophozoites in glass-bottomed culture dishes were covered with a thin layer of LB top agar to restrict parasite movement as described above, and the photobleaching and fluorescence intensity measurements were automated using a Zeiss 510 META confocal microscope and Zeiss LSM software (version 4.2). For photobleaching, the 488-nm laser line was used at maximum output for 20 iterations. A ×63, 1.4-numerical-aperture objective was used with the pinhole set to produce optical sections that were approximately 2 μm thick. Two prebleach and two recovery images were acquired during each cycle, with a 2-s delay between cycles and a total of 80 cycles per sample.

Figure 4B shows images from one representative FLIP experiment. Fluorescence was rapidly lost from the entire bleached cell, consistent with the presence of the GFP-tagged protein in a single compartment. The observed fluorescence loss was specific and not an artifact of repeated imaging, since minimal loss of fluorescence occurred in the control cell. The fluorescence measurements for test and control cells were normalized to their prebleach levels (defined as 100% relative intensity), and their means were plotted versus time to enable statistical analysis of multiple experiments (Fig. 4C). This analysis confirmed the visual result, with the fluorescence loss becoming highly statistically significant at the second bleach cycle and remaining so thereafter ($P \leq 0.0005$ compared to the values for the control; $n = 9$). We concluded that the FLAG-GFP-KDEL protein resided in a single, continuous compartment when expressed in *E. histolytica*.

The major conclusion of this work is that *Entamoeba histolytica* has a continuous ER. This conclusion is in conflict with earlier reports that *E. histolytica* lacks a continuous ER compartment, which were based on work conducted with fixed amebic trophozoites (6, 8, 11, 13, 16). The inability to detect a continuous ER compartment in prior studies presumably was an artifact of fixation. The data supporting the presence of a continuous ER compartment in *E. histolytica* trophozoites are

strong and include the following: (i) the colocalization of the ER-targeted GFP fusion with the ER chaperone BiP in fixed cells, suggesting that the construct was working properly and that the observed compartment was truly the ER; (ii) the apparent restriction of the FLAG-GFP-KDEL protein within a membrane-enclosed compartment, since it was excluded from pseudopodia; (iii) the presence of the FLAG-GFP-KDEL fusion protein within a broadly distributed reticular network with readily visible three-way junctions, which are characteristic of the ER; and (iv) rapid fluorescence loss from the entire cell when one region of the cell was bleached repeatedly. In addition to being important with regard to *E. histolytica* cell biology, these data have important evolutionary implications. The presence of a continuous ER in *E. histolytica* is consistent with the divergence of this parasite from other eukaryotic lineages relatively late in evolution, a notion supported by a growing abundance of molecular data.

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