

**Fig. 4.** Effects of *EhRab11B* overexpression on intracellular and secreted cysteine protease activity.

**A.** Overexpression of *EhRab11B* verified by immunoblotting using anti-HA and anti-*EhRab11B* antibodies. Approximately 20  $\mu$ g of total lysate from the control or HA-*EhRab11B*-overexpressing transformant was electrophoresed on a SDS-polyacrylamide gel under reducing conditions and subjected to an immunoblot analysis using anti-*EhRab11B*, anti-HA and anti-*EhADH* (control). White and black arrow indicate endogenous and HA-tagged *EhRab11B* respectively.

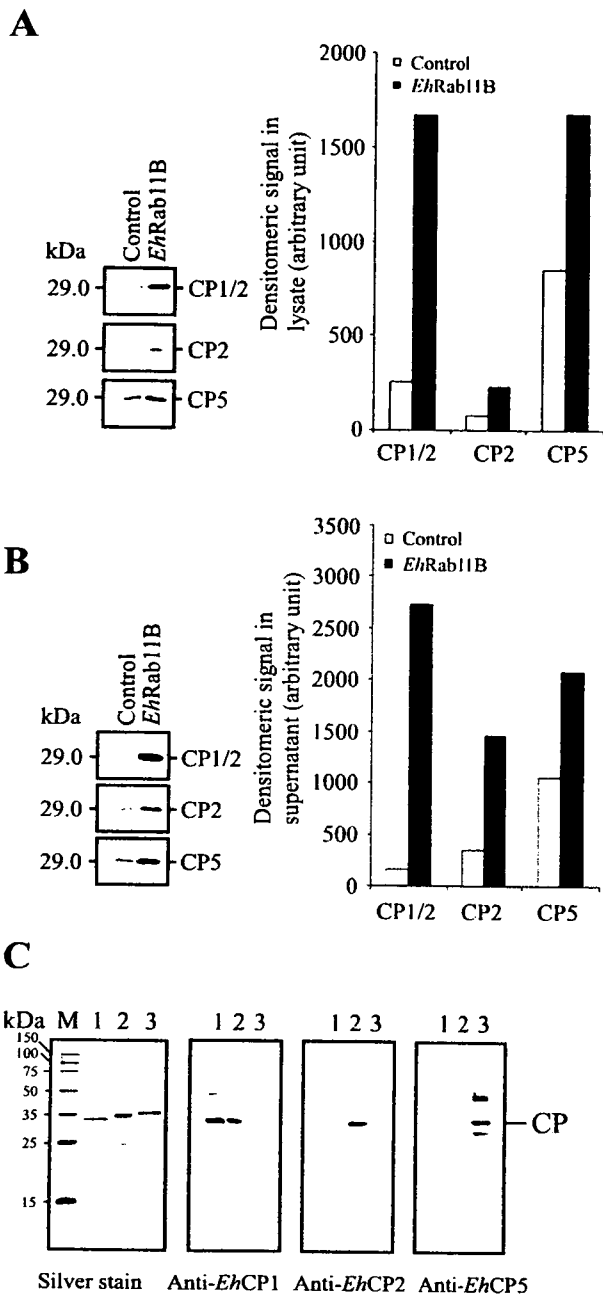
**B and C.** The cysteine protease activity in the total lysate (**B**) and culture supernatant (**C**) of the transformants was measured using z-Arg-Arg-7-amino-4-trifluoromethylcoumarin as a substrate. The level of activity is shown as a percentage relative to the control transformant. Error bars represent SEs of three independent experiments.

**D and E.** Gelatin substrate gel electrophoresis. Approximately 1  $\mu$ g of lysate (**D**) or 12  $\mu$ l of culture supernatant (**E**) per lane was electrophoresed on a 12% SDS polyacrylamide gel copolymerized with 0.1% gelatin.

**F and G.** To evaluate the effect of BFA on cysteine protease activity, the transformants were pretreated with 200  $\mu$ M of BFA for 2 h, and 4  $\mu$ g of lysate (**F**) and 14  $\mu$ l of crude culture supernatant (**G**) were subjected to gelatin substrate gel electrophoresis as in **D** and **E**.

above. That overexpression of *EhRab11B* increased the intracellular and secreted amounts of all three cysteine proteases suggests that *EhRab11B* is involved in the transport of these major cysteine proteases. To ensure cysteine protease production *per se* was not affected by *EhRab11B* overexpression, the steady-state amount of the mRNA of three major cysteine proteases (CP1, CP2 and CP5) as well as an irrelevant control enzyme, methionine  $\gamma$ -lyase 2, was measured by quantitative RT-PCR. The steady-state level of CP1, CP2 or CP5 mRNA, when normalized against methionine  $\gamma$ -lyase 2 mRNA, remained unchanged in the *EhRab11B*-overexpressing transformant compared with the control transformant (at 86%, 70% or 98% of the mRNA level of the control, respectively;  $p > 0.05$ ; data not shown).

Finally, to exclude the possibility that the increased cysteine protease activity in the culture medium of the *EhRab11B*-overexpressing transformant is due to lysed cells, we measured alcohol dehydrogenase (ADH) activity in the culture supernatant and the cell lysate of the *EhRab11B*-overexpressing and control transformants. The ADH activity released into the culture medium by  $2 \times 10^6$  *EhRab11B*-overexpressing and control transformant trophozoites for 1 h was  $2.2 \times 10^{-3}$  and  $1.2 \times 10^{-3}$  U per ml of culture supernatant, respectively, while the ADH activity in the lysate of these transformants was  $1.17 \times 10^{-2}$  and  $1.00 \times 10^{-2}$  U per mg protein respectively. Thus, the 15-fold increase in secreted cysteine protease activity observed for the *EhRab11B*-overexpressing transformant



**Fig. 5.** Immunoblot analysis of intracellular and secreted CP1, CP2 and CP5.

A and B. Approximately 10  $\mu$ g of total lysate (A) or 10  $\mu$ l of concentrated culture supernatant (B) was electrophoresed on a 12% SDS-polyacrylamide gel, and subjected to an immunoblot assay with antibodies raised against recombinant *EhCP1*, *EhCP2* and *EhCP5*. The densitometric quantification of each cysteine protease, shown in the right panels (A and B), was performed by Quantity software version 4.2.1 and the level of cysteine proteases was expressed in arbitrary units.

C. Specificity and cross reactivity of cysteine protease antibodies. Approximately 150 ng of affinity-purified histidine-tagged recombinant CP1 (lane 1), CP2 (lane 2) or CP5 (lane 3) was electrophoresed and subjected to either silver staining (C, left panel) or an immunoblot assay using anti-*EhCP1*, *EhCP2* and *EhCP5* antibodies.

was not attributable to a subtle increase in the lysis of this transformant.

#### *EhRab11B*-overexpressing trophozoites showed an augmented disruption of CHO monolayers

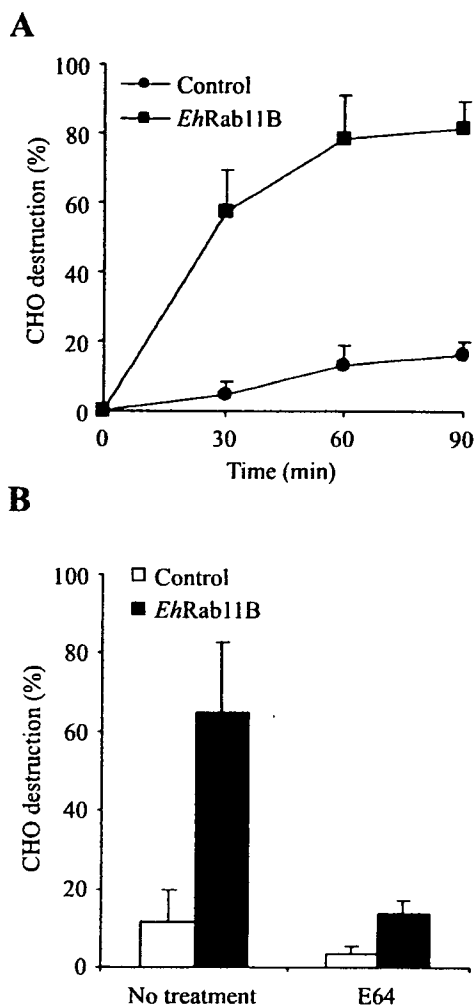
It has been well established that both amoeba extracts and live trophozoites degrade monolayers of cultured mammalian cells (Hellberg *et al.*, 2001; Mitra *et al.*, 2005; 2006; Tillack *et al.*, 2006) and cysteine proteases are primarily responsible for this cytopathic effect (Reed *et al.*, 1993). We examined whether the increased secretion of cysteine proteases caused by the overexpression of *EhRab11B* influences the destruction of CHO monolayers (Fig. 6A). The *EhRab11B*-overexpressing transformant killed CHO cells more efficiently than the control transformant. For example, the *EhRab11B*-overexpressing transformant destroyed  $81.0 \pm 8.0\%$  CHO cells in 90 min, while the control transformant destroyed only  $16.0 \pm 3.7\%$ . Preincubation of *EhRab11B*-overexpressing and control transformants with 200  $\mu$ M E64 for 60 min reduced the extent to which the CHO monolayer was destroyed by 70–75% (Fig. 6B), suggesting that in addition to cysteine proteases, other hydrolases are also involved in the destruction of CHO cells, which is consistent with previous results (Hellberg *et al.*, 2001).

#### Overexpression of *EhRab11B* enhanced the release of a fluid-phase marker

As Rab11 has been implicated in the recycling of endosomes in yeast and mammals (Benli *et al.*, 1996; Ullrich *et al.*, 1996; Ren *et al.*, 1998), effects of the overexpression of *EhRab11B* on the exocytosis of an incorporated fluid-phase marker were investigated. We measured the release of the pH-insensitive fluid-phase marker RITC-dextran by monitoring the decrease in the intracellular RITC fluorescence of transformants preloaded with RITC-dextran and cultured in a marker-free medium. The release of the fluid-phase marker was 1.5–2.0-fold faster in the *EhRab11B*-overexpressing transformant than the control transformant (Fig. 7). The magnitude of the enhancement of exocytosis of the fluid-phase marker by *EhRab11B* overexpression was much lower than that of the oversecretion of cysteine proteases. This agrees well with limited cross-talk between the endocytic pathway and *EhRab11B*, which was demonstrated by the partial colocalization of dextran/*EhRab11B* during endocytosis (Fig. 2).

#### Discussion

In the present study, we have demonstrated for the first time that one of the four Rab11 isoforms plays a crucial role in virulence, in particular the regulation of transport



**Fig. 6.** Effects of *EhRab11B*-overexpression on the destruction of CHO monolayers.

**A.** Kinetics of CHO cell killing by the *EhRab11B*-overexpressing and control transformants. Approximately  $1.5 \times 10^6$  trophozoites of the *EhRab11B*-overexpressing and control transformants were added to a monolayer of CHO cells and incubated at 35°C for the period indicated.

**B.** Effects of E64 on CHO cell killing. The *EhRab11B*-overexpressing and control transformants were pretreated with 200  $\mu$ M of E64 for 2 h and incubated with CHO cells for 60 min. Monolayer destruction is expressed as the percentage of destroyed cells. Data shown here are the mean and standard deviation of three independent experiments.

and secretion of a well-established virulence factor, in enteric protozoan parasites. We have shown that overexpression of *EhRab11B* caused a dramatic increase in secreted as well as, to a lesser degree, intracellular cysteine protease activity, and thereby enhanced *in vitro* cytolytic activity.

Although Rab11 has been implicated in the secretion of several essential cellular factors, the present study is the first to show that Rab11 is directly involved in the pathogenesis of protozoan parasites. It was previously shown

that Rab11 acts as a component of the exocytic machinery in  $Ca^{2+}$ -triggered and constitutive secretion in neuroendocrine and non-neuronal cells (Khvotchev *et al.*, 2003). In mammalian adipocytes, Rab11, together with Arf6, plays a crucial role in the constitutive and insulin-stimulated secretion of one of the adipokines, ACRP30 (Clarke *et al.*, 2006), suggesting that the recycling of endosomes via Rab11 plays an important role in this process. It has also recently been shown that dual Rab11/Arf-binding proteins, FIP3 and 4, function in the delivery of the recycling endosomes to the site of cleavage (cleavage furrow) during cytokinesis by coupling Rab11-positive vesicles from recycling endosomes with the cleavage furrow via interaction with Arf6 and the Exocyst (Fielding *et al.*, 2005).

We have previously shown that the transport and secretion of cysteine proteases are regulated through the specific interaction of *EhRab7A* with a novel effector called the retromer-like complex, composed of Vps26, Vps29 and Vps35 (Nakada-Tsukui *et al.*, 2005). Although the interaction of *EhRab7A* with the retromer-like complex is involved in part in the regulation of transport and secretion, the fold increase in intracellular and secreted levels of cysteine proteases caused by overexpression of *EhRab7A* is much less than that observed on overexpression of *EhRab11B*, shown in this study. These data suggest that *EhRab11B* plays a pivotal role in the trafficking of cysteine proteases. Alternatively, the remarkable changes in cysteine protease activity caused by *EhRab11B* overexpression might be attributable to the high level of overexpression of *EhRab11B* (ninefold), compared with only an approximately twofold increase in the case of overexpression of *EhRab7A* and *EhRab5*.

Although among the three cysteine proteases examined CP1 appeared to be influenced most dramatically by *EhRab11B* overexpression, *EhRab11B* mediates BFA-insensitive transport of all three major cysteine proteases and there is no stringent specificity in the *EhRab11B*-mediated pathway (Figs 3 and 4). It has recently been reported that overexpression of CP5 causes concomitant augmentation of the amount and activity of both CP1 and CP2 (Tillack *et al.*, 2006), which may suggest that CP5 is a key processing protease of other cysteine proteases. It is conceivable that similarly, activation of *EhRab11B*-mediated transport enhances the activity of a key processing enzyme, which in turn results in an augmented expression of various cysteine proteases. Although the pore-forming peptide amoebapore and the surface galactose/*N*-acetylgalactosamine-specific lectin were implicated in virulence *in vitro* and *in vivo* (Vines *et al.*, 1998; Leippe, 1999), it has not been shown in the present study if the transport and secretion of these proteins as well as other hydrolytic proteins are also mediated by *EhRab11B*. However, it is unlikely that *EhRab11B* mediates the transport of plasma membrane proteins because

the *EhRab11B*-mediated transport pathway is resistant to BFA while the galactose/N-acetylgalactosamine-specific lectin was shown to be transported via the 'classical' BFA-sensitive pathway (Manning-Cela *et al.*, 2003). It needs to be determined if other secreted virulence factors including amoebapore are also transported via an *EhRab11B*-associated pathway. In mammalian cells, GBF1, an Arf guanine-nucleotide exchange factor (GEF), was shown to be resistant to BFA and displayed specificity for Arf5, not but Arf1, the latter of which was catalysed by BFA-sensitive Arf GEF (Claude *et al.*, 1999). *E. histolytica* encodes 10 Arf proteins (Clark *et al.*, 2007) and nine Arf GEF-like proteins (Y. Saito-Nakano and T. Nozaki, unpublished), which raises the possibility that sensitivity to BFA may vary among these Arf-GEFs and the secretion of cysteine proteases is regulated by a pathway involving a BFA-insensitive Arf-GEF in *E. histolytica*.

The simultaneous increase of cysteine protease activity in both the whole lysate and the culture medium caused by *EhRab11B* overexpression has not been well explained. There are at least a few possible explanations. First, cysteine proteases that are usually destined to be transported to the degradation compartment were redirected to be accumulated in the storage compartment and also trafficked via the secretory pathway. This may be a consequence of either the ablation or enhancement of post-translational modifications by *EhRab11B* overexpression. Second, overexpression of *EhRab11B* activated an unidentified key protease responsible for the processing and activation of various cysteine proteases. It needs to be carefully examined if the simple overexpression of individual cysteine proteases increases secreted as well as intracellular amounts of cysteine proteases. It was previously shown that overexpression of three cysteine proteases, CP1, CP2 and CP5, caused a three- to sixfold increase in the total (i.e. intracellular) amount of cysteine proteases (Hellberg *et al.*, 2001; Tillack *et al.*, 2006). In the latter study, it was also shown that overexpression of CP1 or CP2 increased only the activity of a corresponding cysteine protease while overexpression of CP5 increased that of all three cysteine proteases. This observation may be causally connected with the finding that overexpression of CP1 or CP2 caused only a slight (1.5- to twofold) increase in the destruction of CHO cells whereas overexpression of CP5 augmented the killing by fivefold. However, it was not demonstrated in these studies whether overexpression of cysteine proteases also specifically or non-specifically increased the secretion of cysteine proteases.

A line of evidence suggested that *EhRab11B* is localized to the TGN and/or the post-Golgi compartments that interact with the endocytic pathway. *EhRab11B* partially colocalized with late endosomes (Fig. 2). In addition, the overexpression of *EhRab11B* dramatically activated the

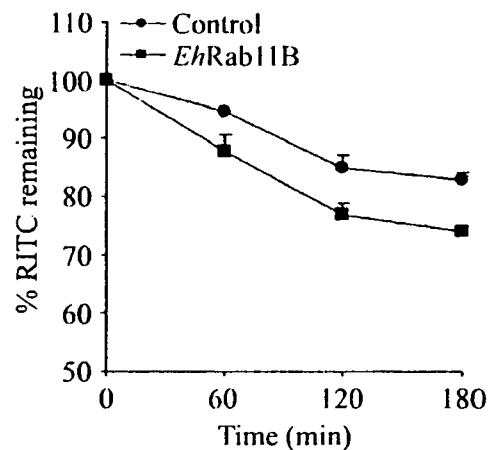


Fig. 7. Overexpression of *EhRab11B* affected exocytosis of the fluid-phase marker. The *EhRab11B*-overexpressing and control transformants were labelled with the fluid-phase marker RITC-dextran for 3 h, and chased for 180 min. At the time indicated, cells were collected and washed, and the fluorescence of RITC-dextran was measured using a fluorometer. The remaining fluorescence is shown as a percentage relative to the no-chase control.

trafficking and secretion of cysteine proteases (Figs 3 and 4), and also slightly enhanced the exocytosis of the internalized fluid-phase marker (Fig. 7). These results also indicate that *EhRab11B* may control trafficking at the intersection of endocytic and secretory pathways in this organism. In contrast to *EhRab11B*, *EhRab11A* is not likely involved in endocytosis. Although *EhRab11A* was concentrated in the early and late endosome-enriched fractions (Temesvari *et al.*, 1999), *EhRab11A* was not colocalized with markers for fluid-phase and receptor-mediated endocytosis (McGugan and Temesvari, 2003). Recently, *EhRab11A* was shown to be translocated to the periphery of the cell under iron and serum starvation and during encystation, suggestive of its possible involvement in the secretory pathway under stress and during differentiation (Manning-Cela *et al.*, 2003). Therefore, we propose that *EhRab11A* and *EhRab11B* have distinct non-redundant functions, which need to be unequivocally demonstrated. Finally, considering that most organelles in *E. histolytica* are not morphologically discernible, 'classical' structures (Martinez-Palomo, 1986; Mazzucco *et al.*, 1997), the present study describing the unique involvement of Rab11 in the pathogenicity of this organism might illuminate the functional divergence of the secretory pathways in eukaryotes.

## Experimental procedures

### Chemical and reagents

All chemicals of analytical grade were purchased from Wako (Tokyo, Japan) unless otherwise stated. Trans-epoxysuccinyl-L-leucylamido-(4-guanidino) butane (E64) was obtained from

Sigma-Aldrich (Tokyo, Japan). 2',7'-bis(2-carboxyethyl)-5(6)-carboxyfluorescein acetoxymethyl ester, BFA, CellTracker Blue (7-amino-4-chloromethylcoumarin), LysoTracker Green DND 26, and LysoTracker Red DND-99 were purchased from Invitrogen (Carlsbad, CA).

### Organisms and culture

Trophozoites of *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). For the detection of proteins in the lysates and culture supernatant, approximately  $1 \times 10^6$  trophozoites of the late-logarithmic growth phase were cultivated in 1 ml of serum-free BI-S-33 medium under anaerobic conditions using Anaerocult A (Merck, Darmstadt, Germany) on a 6-well culture plate (Corning, NY) at 35°C for 2 h. CHO cells were grown in F-12 medium (Invitrogen-Gibco, Grand Island, NY) supplemented with 10% fetal bovine serum on a 10-cm-diameter tissue culture dish (IWAKI, Tokyo, Japan) under 5% CO<sub>2</sub> at 37°C.

### Quantitative RT-PCR

Poly-A RNA was extracted from HM1: IMSS cl6 trophozoites with a Messenger RNA Isolation Kit (Stratagene), and treated with deoxyribonuclease I (Invitrogen) to exclude genomic DNA. Poly-A RNA was reverse transcribed with the SuperScript III First-Strand Synthesis System and oligo(dT)<sub>20</sub> primer (Invitrogen). PCR was performed with the resulting cDNA as a template and specific oligonucleotide primers using the ABI PRISM 7700 Sequence Detection System (Applied Biosystems, Japan). Primers used were 5'-GTT ATT ACA AAA GAA GAA GCA GAA-3' and 5'-TCA GCAACAACCTTT TTT GTT CTC-3' (*EhRab5*); 5'-GCT GAACAA TGG TGT TCA GAA CAT-3' and 5'-TCAACAAGG GCAATC AGA CTT TTG-3' (*EhRab7A*); 5'-TGT CAA AAA CAA AAT CTC TTT TTC-3' and 5'-TTA GCA ACA TCC TCC TTT ATT TTC-3' (*EhRab11A*); 5'-AAT AAT GCA TTC TTT TTT GAA ACA-3' and 5'-TTA ACA ACA TTT CTT TTC TTC TGG-3' (*EhRab11B*); 5'-TTC TTT ATT GAA ACT TCT GCT TTA-3' and 5'-AAT ACA GCA CTT CTT ACC ACC TTG-3' (*EhRab11C*); 5'-TTC TTT TTT GAA ACA TCA GCT TTT-3' and 5'-TTAACAAGC ACAACC ACT TTT TTT-3' (*EhRab11D*); 5'-CAA TGC AAG AAT AAC TAT TTT GCA-3' and 5'-TCA GAG ATA TTC AAC ACC AGT TGG-3' (CP1); 5'-AAA TGC AAG AAT AAC TAC TTT GCT-3' and 5'-TCA AAG ATA TTG AAC GCC AGT TGG-3' (CP2); 5'-AAA GAA TGT TCA TCA ACT CAG CTT-3' and 5'-TTA AGC ATC AGC AAC CCC AAC TGG-3' (CP5); and 5'-CAT GTT AAA TTA TGT GGA TTA GCT-3' and 5'-TCAATT ATT TTC TGA CCC GTC TTC-3' (methionine  $\gamma$ -lyase 2); 5'-GAT CCA ACA TAT CCT AAA ACA ACA-3' and 5'-TCA ATT ATT TTC TGA CCC GTC TTC-3' (RNA polymerase II 15 kDa subunit). Parameters for PCR were: 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 primer dimers.

### Production of *E. histolytica* transformants overexpressing EhRab11B

The *EhRab11B* protein coding region was amplified by PCR from cDNA using sense and antisense oligonucleotides containing

appropriate restriction sites at the 5'-end, 5'-GA AGATCT A ATG GCTAGC GGA TCA GAA GAA TAT GAT TTC-3' and 5'-CCA CTCGAG TTA ACA ACA TTT CTT TTC TTC TGG-3' (restriction sites are underlined). A sequence tag consisting of three tandem repeats of the haemagglutinin (HA) peptide was inserted at the amino terminus together with an engineered NheI site (Saito-Nakano *et al.*, 2004). A PCR-amplified DNA fragment was digested with BglII and XhoI, and ligated into BglII and XhoI sites of the expression vector pEhEx, to produce pH 11B. The wild-type trophozoites were transformed with either pH 11B or pEhEx by liposome-mediated transfection as previously described (Nozaki *et al.*, 1999).

### Assay for destruction of CHO monolayers

The destruction of CHO monolayers was quantified as described previously with slight modifications (Mitra *et al.*, 2006). Briefly, CHO cells were labelled with 40  $\mu$ M of CellTracker Blue in the growth medium at 37°C for 2 h, and then washed and chased in the growth medium for 2 h. After the medium was replaced with prewarmed OPTI-MEM (Invitrogen-Gibco) supplemented with 137 mM L-cysteine and 19 mM ascorbic acid, pH 6.7, approximately  $1.5 \times 10^5$  *E. histolytica* trophozoites were added and incubated at 35°C for 0, 30, 60 and 90 min. After this incubation, the remaining CHO cells were collected using trypsin and the fluorescence of CellTracker blue was measured using a fluorometer (F-2500, Hitachi, Japan) with excitation and emission at 353 and 465 nm respectively. The number of adherent CHO cells was proportional to the intensity of CellTracker blue staining and expressed as a percentage of the remaining fluorescence of untreated CHO cells.

### Preparation of cell lysates and culture supernatants

A semi-confluent culture was harvested at 48–72 h after initiation of the culture and suspended in the serum-free BI-S-33 medium at  $1 \times 10^6$  cells ml<sup>-1</sup>. After the culture was incubated at 35°C for 2 h, the culture supernatant was centrifuged at 15 000 g for 10 min at 4°C. Attached trophozoites were washed with ice-cold phosphate-buffered saline (PBS) containing 2% glucose and collected by centrifugation at 1000 g for 10 min. For immunoblot analysis, the culture supernatant was concentrated by 10-fold with TCA-acetone precipitation. For soluble extracts, approximately  $2.5 \times 10^5$  trophozoites lysed in 100  $\mu$ l of PBS by three cycles of freeze-thawing were centrifuged at 14 000 g for 15 min at 4°C, and the supernatant was saved.

### Assay for cysteine protease activity

Cysteine protease activity was measured with the cleavage of the synthetic peptide substrate z-Arg-Arg-7-amino-4-trifluoromethylcoumarin (ICN, Aurora, OH) monitored by a spectrophotometric method as described previously (Nowak *et al.*, 2004; Nakada-Tsukui *et al.*, 2005). The activities were expressed in mmol of z-Arg-Arg-7-amino-4-trifluoromethylcoumarin produced per milligram of lysate protein. The statistical significance of the data was evaluated with Student's *t*-test.

### Assay for ADH activity

The ADH activity in the cell lysate and the culture supernatant was determined by monitoring the increase in absorbance at

334 nm due to the reduction of NADP<sup>+</sup> with a spectrophotometer (Ultraspec 3000, Pharmacia Biotech) in 200 µl of 50 mM glycine-NaOH (pH 9.5) containing 250 µM NADP<sup>+</sup> and 125 mM ethanol at 35.5°C for 1 h, essentially as described (Kumar *et al.*, 1992). Approximately  $5 \times 10^5$  trophozoites were incubated in 250 µl of OPTI-MEM (see above) at 37°C for 1 h. One unit of activity was defined as the amount of enzyme that produced 1 µmol of NADPH per min at 35.5°C.

#### Substrate gel electrophoresis

Protease activity was assayed by gelatin substrate gel electrophoresis as described previously (Reed *et al.*, 1989; Hellberg *et al.*, 2000). Briefly, 12 µl of the crude culture supernatant or 1–4 µg of the lysate was electrophoresed under non-reducing conditions on a 12% SDS-polyacrylamide gel copolymerized with 0.1% gelatin (Sigma-Aldrich, Japan). After separation, the gel was washed with 2.5% Triton X-100 to remove SDS for 1 h, rinsed twice with distilled water, and incubated in the developing buffer (100 mM Sodium acetate, 1% Triton X-100, 2 mM DTT) at room temperature for 3 h. After staining and several cycles of destaining, protease activity was detected by the degradation of gelatin, indicated by a clear band on the Coomassie brilliant blue-stained background. Molecular weight was estimated from the migration of standards (Bio-Rad Laboratories, Richmond, CA).

#### Production and purification of recombinant cysteine proteases

*EhCP1*, *EhCP2* and *EhCP5* genes lacking putative signal sequences were amplified by PCR using trophozoite cDNA as a template and oligonucleotide primers designed based on available nucleotide sequences: Q01957 (CP1), Q01958 (CP2) and AAFB01000593 (CP5). PCR products were inserted into the pET-15b expression plasmid (Novagen). Histidine-tagged recombinant cysteine proteases were produced as inclusion bodies in *E. coli* strain BL21 (DE3). Recombinant cysteine proteases were purified by affinity chromatography under denaturing conditions. Refolding and activation were performed as previously described (Hellberg *et al.*, 2002; Sato *et al.*, 2006) with some modifications. Briefly, recombinant CP1 and CP2 (100 µg ml<sup>-1</sup>, 30–40 µl) were dialysed against refolding buffer B (100 mM Tris-HCl, pH 8.8, 300 mM NaCl, 5% glycerol, 0.1 mM EDTA, 3 mM reduced glutathione and 0.5 mM oxidized glutathione) at 4°C for 1.5 h, and subsequently against refolding buffer C (25 mM Tris-HCl, pH 6.8 and 150 mM NaCl) at 4°C for 1.5 h. Recombinant CP5 was treated similarly except that refolding buffer B contained 50 mM Tris-HCl, pH 8.8 and refolding buffer C contained 50 mM Tris-HCl, pH 6.8 and no NaCl. The refolded recombinant cysteine proteases were activated by incubation with an equal volume of 100 mM Tris-HCl, pH 8.8, 20 mM DTT and 2% Triton X-100 at 37°C for 30 min.

#### Antibodies

Affinity-purified anti-*EhCP1*, anti-*EhCP2*, anti-*EhCP5* and anti-*EhRab11B* rabbit polyclonal antibodies were commercially produced at Kitayama-Rabes (Nagano, Japan). Anti-HA 16B12 mouse monoclonal antibody was purchased from Berkeley Antibody (Berkeley, CA). Anti-*EhADH* rabbit polyclonal antibody was described previously (Sanuki *et al.*, 2001). Anti-*EhSec61* α-subunit and anti-DPMS rabbit polyclonal antibodies were a gift

from Rosana Sánchez-López. Alexa Fluor anti-mouse and anti-rabbit IgG, and horseradish peroxidase (HRP)-conjugated goat anti-mouse and anti-rabbit IgG were purchased from Invitrogen (Carlsbad, CA).

#### Immunoblot analysis

Cell lysates and culture supernatants were separated on 12% (w/v) SDS-polyacrylamide gels and subsequently electrotransferred onto nitrocellulose membranes (Hybond-C Extra; Amersham Biosciences UK, Little Chalfont, Bucks, UK) essentially as described (Tokoro *et al.*, 2003). Non-specific binding was blocked by incubating the membranes for 1.5 h at room temperature in 5% non-fat dried milk in TBST (50 mM Tris-HCl, pH 8.0, 150 mM NaCl and 0.05% Tween-20). The blots were reacted with primary antibodies specific for *EhCP1*, *EhCP2*, *EhCP5*, *EhRab11B*, *EhADH* (control) and HA 16B12 at a dilution of 1:40 to 1:1000. The membranes were washed with TBST and further reacted with HRP-conjugated anti-rabbit or anti-mouse IgG antisera (1:10 000–1:20 000) at room temperature for 1.5 h. After further washing with TBST, specific proteins were visualized with chemiluminescence detection (Millipore Corporation, Billerica, MA). Densitometric measurements were performed using Quantity One software (version 4.2.1, Bio-Rad Laboratories, Hercules, CA).

#### Indirect immunofluorescence

The trophozoites were incubated with 2 mg ml<sup>-1</sup> of FITC-dextran in BI-S-33 medium for 1 h, washed, and further incubated in fresh prewarmed BI-S-33 medium for specific periods (0, 30, 60 and 120 min). Cells were transferred to 8-mm round wells on a slide glass, fixed with 3.7% paraformaldehyde, and permeabilized with 50 mM digitonin/PBS as previously described (Saito-Nakano *et al.*, 2004). The cells were then reacted with anti-HA 16B12 mouse monoclonal antibody (1:1000) and Alexa Fluor-568 anti-mouse secondary antibody (1:1000). To examine the subcellular localization of *EhRab11B* in ER, anti-*EhSec61* α-subunit or anti-*EhDPMS* antibody (1:1000) was used. The samples were examined on a Carl-Zeiss LSM510 confocal laser-scanning microscope (Thornwood, NY). Images were further analysed using LSM510 software.

#### Measurement of fluid-phase exocytosis

To measure fluid-phase exocytosis,  $5 \times 10^5$  amoebic transformants were cultured in BI-S-33 medium containing the pH-insensitive fluorescent fluid-phase marker RITC-dextran (2 mg ml<sup>-1</sup>; Mr = 70 000; Sigma-Aldrich, Japan) at 35°C for 3 h to saturate all endocytic compartments with the marker. The labelled cells were collected, washed and resuspended in warm marker-free BI-S-33 medium, and incubated at 35°C for 3 h. At specific time points (0, 60, 120 and 180 min), cells were collected and washed three times with ice-cold PBS. The cell pellets were then suspended in 300 µl of 50 mM Tris-HCl, pH 7.0 containing 1% Triton-X100 and vortexed for 15 s. Fluorescence intensity was measured using fluorometer (F-2500, Hitachi, Japan) at excitation and emission wavelengths of 570 and 610 nm respectively.

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## References

- Ankri, S., Stolarsky, T., Bracha, R., Padilla-Vaca, F., and Mirelman, D. (1999) Antisense inhibition of expression of cysteine proteinases affects *Entamoeba histolytica*-induced formation of liver abscess in hamsters. *Infect Immun* **67**: 421–422.
- Benli, M., Doring, F., Robinson, D.G., Yang, X., and Gallwitz, D. (1996) Two GTPase isoforms, Ypt31p and Ypt32p, are essential for Golgi function in yeast. *EMBO J* **15**: 6460–6475.
- Bock, J.B., Matern, H.T., Peden, A.A., and Scheller, R.H. (2001) A genomic perspective on membrane compartment organization. *Nature* **409**: 839–841.
- Bruchhaus, I., Jacobs, T., Leippe, M., and Tannich, E. (1996) *Entamoeba histolytica* and *Entamoeba dispar*: differences in numbers and expression of cysteine proteinase genes. *Mol Microbiol* **22**: 255–263.
- Chavrier, P., and Goud, B. (1999) The role of ARF and Rab GTPases in membrane transport. *Curr Opin Cell Biol* **11**: 466–475.
- Chen, Y.A., Scales, S.J., Patel, S.M., Doung, Y.C., and Scheller, R.H. (1999) SNARE complex formation is triggered by Ca<sup>2+</sup> and drives membrane fusion. *Cell* **97**: 165–174.
- 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.
- Clark, C.G., Cecilia, U., Alsmark, M., Hofer, M., Saito-Nakano, Y., Ali, V., et al. (2007) Structure and content of the *Entamoeba histolytica* genome. *Adv Parasitol* (in press).
- Clarke, M., Ewart, M.A., Santy, L.C., Prekeris, R., and Gould, G.W. (2006) ACRP30 is secreted from 3T3-L1 adipocytes via a Rab11-dependent pathway. *Biochem Biophys Res Commun* **342**: 1361–1367.
- Claude, A., Zhao, B.P., Kuziemy, C.E., Dahan, S., Berger, S.J., Yan, J.P., et al. (1999) GBF1: a novel Golgi-associated BFA-resistant guanine nucleotide exchange factor that displays specificity for ADP-ribosylation factor 5. *J Cell Biol* **146**: 71–84.
- Diamond, L.S., Mattern, C.F., and Bartgis, I.L. (1972) Viruses of *Entamoeba histolytica*. I. Identification of transmissible virus-like agents. *J Virol* **9**: 326–341.
- 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.
- Eichinger, D. (1997) Encystation of *Entamoeba* parasites. *BioEssays* **19**: 633–639.
- Fader, C.M., Savina, A., Sanchez, D., and Colombo, M.I. (2005) Exosome secretion and red cell maturation: exploring molecular components involved in the docking and fusion of multivesicular bodies in K562 cells. *Blood Cells Mol Dis* **35**: 153–157.
- Fielding, A.B., Schonteich, E., Matheson, J., Wilson, G., Yu, X., Hickson, G.R., et al. (2005) Rab11-FIP3 and FIP4 interact with Arf6 and the exocyst to control membrane traffic in cytokinesis. *EMBO J* **24**: 3389–3399.
- Goldenring, J.R., Soroka, C.J., Shen, K.R., Tang, L.H., Rodriguez, W., Vaughan, H.D., et al. (1994) Enrichment of rab11, a small GTP-binding protein, in gastric parietal cells. *Am J Physiol* **267**: G187–G194.
- Harris, E., Yoshida, K., Cardelli, J., and Bush, J. (2001) Rab11-like GTPase associates with and regulates the structure and function of the contractile vacuole system in *Dictyostelium*. *J Cell Sci* **114**: 3035–3045.
- Hellberg, A., Leippe, M., and Bruchhaus, I. (2000) Two major 'higher molecular mass proteinases' of *Entamoeba histolytica* are identified as cysteine proteinases 1 and 2. *Mol Biochem Parasitol* **105**: 305–309.
- Hellberg, A., Nickel, R., Lotter, H., Tannich, E., and Bruchhaus, I. (2001) Overexpression of cysteine proteinase 2 in *Entamoeba histolytica* or *Entamoeba dispar* increases amoeba-induced monolayer destruction *in vitro* but does not augment amoebic liver abscess formation in gerbils. *Cell Microbiol* **3**: 13–20.
- Hellberg, A., Nowak, N., Leippe, M., Tannich, E., and Bruchhaus, I. (2002) Recombinant expression and purification of an enzymatically active cysteine proteinase of the protozoan parasite *Entamoeba histolytica*. *Protein Expr Purif* **24**: 131–137.
- Hume, A.N., Collinson, L.M., Rapak, A., Gomes, A.Q., Hopkins, C.R., and Seabra, M.C. (2001) Rab27a regulates the peripheral distribution of melanosomes in melanocytes. *J Cell Biol* **152**: 795–808.
- Jacobs, T., Bruchhaus, I., Dandekar, T., Tannich, E., and Leippe, M. (1998) Isolation and molecular characterization of a surface-bound proteinase of *Entamoeba histolytica*. *Mol Microbiol* **27**: 269–276.
- Jeffries, T.R., Morgan, G.W., and Field, M.C. (2001) A developmentally regulated rab11 homologue in *Trypanosoma brucei* is involved in recycling processes. *J Cell Sci* **114**: 2617–2626.
- Khvotchev, M.V., Ren, M., Takamori, S., Jahn, R., and Sudhof, T.C. (2003) Divergent functions of neuronal Rab11b in Ca<sup>2+</sup>-regulated versus constitutive exocytosis. *J Neurosci* **23**: 10531–10539.
- Kumar, A., Shen, P., Descoteaux, S., Pohl, J., Bailey, G., and Samuelson, J. (1992) Cloning and expression of an NADP<sup>+</sup>-dependent alcohol dehydrogenase gene of *Entamoeba histolytica*. *Proc Natl Acad Sci USA* **89**: 10188–10192.
- Lal, K., Field, M.C., Carlton, J.M., Warwicker, J., and Hirt, R.P. (2005) Identification of a very large Rab GTPase

- family in the parasitic protozoan *Trichomonas vaginalis*. *Mol Biochem Parasitol* **143**: 226–235.
- Leippe, M. (1999) Antimicrobial and cytolytic polypeptides of amoeboid protozoa-effector molecules of primitive phagocytes. *Dev Comp Immunol* **23**: 267–279.
- Loftus, B., Anderson, I., Davies, R., Alismark, U.C.M., Samuelson, J., Amedeo, P., et al. (2005) The genome of the protist parasite *Entamoeba histolytica*. *Nature* **433**: 865–868.
- 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.
- McNiven, M.A., and Thompson, H.M. (2006) Vesicle formation at the plasma membrane and trans-Golgi network: the same but different. *Science* **313**: 1591–1594.
- Manning-Cela, R., Marquez, C., Franco, E., Talamas-Rohana, P., and Meza, I. (2003) BFA-sensitive and insensitive exocytic pathways in *Entamoeba histolytica* trophozoites: their relationship to pathogenesis. *Cell Microbiol* **5**: 921–932.
- Martinez-Palomo, A. (ed.) (1986) *The Biology of Entamoeba histolytica*, Chichester: Research Studies Press.
- Mazzucco, A., Benchimol, M., and De Souza, W. (1997) Endoplasmic reticulum and Golgi-like elements in *Entamoeba*. *Micron* **28**: 241–247.
- Mitra, B.N., Yasuda, T., Kobayashi, S., Saito-Nakano, Y., and Nozaki, T. (2005) Differences in morphology of phagosomes and kinetics of acidification and degradation in phagosomes between the pathogenic *Entamoeba histolytica* and the non-pathogenic *Entamoeba dispar*. *Cell Motil Cytoskeleton* **62**: 84–99.
- Mitra, B.N., Kobayashi, S., Saito-Nakano, Y., and Nozaki, T. (2006) *Entamoeba histolytica*: differences in phagosome acidification and degradation between attenuated and virulent strains. *Exp Parasitol* **114**: 57–61.
- 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 Biol Cell* **16**: 5294–5303.
- Novick, P., and Zerial, M. (1997) The diversity of Rab proteins in vesicle transport. *Curr Opin Cell Biol* **9**: 496–504.
- Nowak, N., Lotter, H., Tannich, E., and Bruchhaus, I. (2004) Resistance of *Entamoeba histolytica* to the cysteine proteinase inhibitor E64 is associated with secretion of proenzymes and reduced pathogenicity. *J Biol Chem* **279**: 38260–38266.
- 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.
- Que, X., and Reed, S.L. (2000) Cysteine proteinases and the pathogenesis of amebiasis. *Clin Microbiol Rev* **13**: 196–206.
- Que, X., Brinen, L.S., Perkins, P., Herdman, S., Hirata, K., Torian, B.E., et al. (2002) Cysteine proteinases from distinct cellular compartments are recruited to phagocytic vesicles by *Entamoeba histolytica*. *Mol Biochem Parasitol* **119**: 23–32.
- Reed, S., Bouvier, J., Pollack, A.S., Engel, J.C., Brown, M., Hirata, K., et al. (1993) Cloning of a virulence factor of *Entamoeba histolytica*. Pathogenic strains possess a unique cysteine proteinase gene. *J Clin Invest* **91**: 1532–1540.
- Reed, S.L., Keene, W.E., and McKerrow, J.H. (1989) Thiol proteinase expression and pathogenicity of *Entamoeba histolytica*. *J Clin Microbiol* **27**: 2772–2777.
- Ren, M., Xu, G., Zeng, J., De Lemos-Chiarandini, C., Adesnik, M., and Sabatini, D.D. (1998) Hydrolysis of GTP on rab11 is required for the direct delivery of transferrin from the pericentriolar recycling compartment to the cell surface but not from sorting endosomes. *Proc Natl Acad Sci USA* **95**: 6187–6192.
- Saito-Nakano, Y., Nakazawa, M., Shigeta, Y., Takeuchi, T., and Nozaki, T. (2001) Identification and characterization of genes encoding novel Rab proteins from *Entamoeba histolytica*. *Mol Biochem Parasitol* **116**: 219–222.
- Saito-Nakano, Y., Yasuda, T., Nakada-Tsukui, K., Leippe, M., and Nozaki, T. (2004) Rab5-associated vacuoles play a unique role in phagocytosis of the enteric protozoan parasite *Entamoeba histolytica*. *J Biol Chem* **279**: 49497–49507.
- 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., Mitra, 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* (in press).
- Sanuki, J., Nakano, K., Tokoro, M., Nozaki, T., Okuzawa, E., Kobayashi, S., and Asai, T. (2001) Purification and identification of major soluble 40-kDa antigenic protein from *Entamoeba histolytica*: its application for serodiagnosis of asymptomatic amebiasis. *Parasitol Int* **50**: 73–80.
- Saric, M., Vahrmann, A., Bruchhaus, I., Bakker-Grunwald, T., and Scholze, H. (2006) The second cysteine protease inhibitor, EhICP2, has a different localization in trophozoites of *Entamoeba histolytica* than EhICP1. *Parasitol Res* **100**: 171–174.
- 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.
- Savina, A., Vidal, M., and Colombo, M.I. (2002) The exosome pathway in K562 cells is regulated by Rab11. *J Cell Sci* **115**: 2505–2515.
- Schluter, O.M., Khvotchev, M., Jahn, R., and Sudhof, T.C. (2002) Localization versus function of Rab3 proteins. Evidence for a common regulatory role in controlling fusion. *J Biol Chem* **277**: 40919–40929.
- Seabra, M.C., Mules, E.H., and Hume, A.N. (2002) Rab GTPases, intracellular traffic and disease. *Trends Mol Med* **8**: 23–30.
- Somsel Rodman, J., and Wandinger-Ness, A. (2000) Rab GTPases coordinate endocytosis. *J Cell Sci* **113**: 183–192.
- Stanley, S.L., Jr, Zhang, T., Rubin, D., and Li, E. (1995) Role of the *Entamoeba histolytica* cysteine proteinase in amebic



- liver abscess formation in severe combined immunodeficient mice. *Infect Immun* **63**: 1587–1590.
- Stenmark, H., and Olkkonen, V.M. (2001) The Rab GTPase family. *Genome Biol* **2**: REVIEWS3007.
- Stinchcombe, J.C., Barral, D.C., Mules, E.H., Booth, S., Hume, A.N., Machesky, L.M., *et al.* (2001) Rab27a is required for regulated secretion in cytotoxic T lymphocytes. *J Cell Biol* **152**: 825–834.
- Temesvari, L.A., Harris, E.N., Stanley, S.L., Jr and Cardelli, J.A. (1999) Early and late endosomal compartments of *Entamoeba histolytica* are enriched in cysteine proteases, acid phosphatase and several Ras-related Rab GTPases. *Mol Biochem Parasitol* **103**: 225–241.
- Tillack, M., Nowak, N., Lotter, H., Bracha, R., Mirelman, D., Tannich, E., and Bruchhaus, I. (2006) Increased expression of the major cysteine proteinases by stable episomal transfection underlines the important role of EhCP5 for the pathogenicity of *Entamoeba histolytica*. *Mol Biochem Parasitol* **149**: 58–64.
- Tokoro, M., Asai, T., Kobayashi, S., Takeuchi, T., and Nozaki, T. (2003) Identification and characterization of two isoenzymes of methionine  $\gamma$ -lyase from *Entamoeba histolytica*: a key enzyme of sulfur-amino acid degradation in an anaerobic parasitic protist that lacks forward and reverse trans-sulfuration pathways. *J Biol Chem* **278**: 42717–42727.
- Ullrich, O., Reinsch, S., Urbe, S., Zerial, M., and Parton, R.G. (1996) Rab11 regulates recycling through the pericentriolar recycling endosome. *J Cell Biol* **135**: 913–924.
- Urbe, S., Huber, L.A., Zerial, M., Tooze, S.A., and Parton, R.G. (1993) Rab11, a small GTPase associated with both constitutive and regulated secretory pathways in PC12 cells. *FEBS Lett* **334**: 175–182.
- Vines, R.R., Ramakrishnan, G., Rogers, J.B., Lockhart, L.A., Mann, B.J., and Petri, W.A., Jr (1998) Regulation of adherence and virulence by the *Entamoeba histolytica* lectin cytoplasmic domain, which contains a  $\beta$ 2 integrin motif. *Mol Biol Cell* **9**: 2069–2079.
- Wilcke, M., Johannes, L., Galli, T., Mayau, V., Goud, B., and Salamero, J. (2000) Rab11 regulates the compartmentalization of early endosomes required for efficient transport from early endosomes to the trans-golgi network. *J Cell Biol* **151**: 1207–1220.
- Willhoeft, U., Hamann, L., and Tannich, E. (1999) A DNA sequence corresponding to the gene encoding cysteine proteinase 5 in *Entamoeba histolytica* is present and positionally conserved but highly degenerated in *Entamoeba dispar*. *Infect Immun* **67**: 5925–5929.
- Zerial, M., and McBride, H. (2001) Rab proteins as membrane organizers. *Nat Rev Mol Cell Biol* **2**: 107–117.
- Zhang, Z., Wang, L., Seydel, K.B., Li, E., Ankri, S., Mirelman, D., and Stanley, S.L., Jr (2000) *Entamoeba histolytica* cysteine proteinases with interleukin-1 beta converting enzyme (ICE) activity cause intestinal inflammation and tissue damage in amoebiasis. *Mol Microbiol* **37**: 542–548.



## An *Entamoeba* sp. strain isolated from rhesus monkey is virulent but genetically different from *Entamoeba histolytica*<sup>☆</sup>

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### Abstract

An *Entamoeba* sp. strain, P19-061405, was isolated from a rhesus monkey in Nepal and characterized genetically. The strain was initially identified as *Entamoeba histolytica* using PCR amplification of peroxiredoxin genes. However, sequence analysis of the 18S rRNA gene showed a 0.8% difference when compared to the reference *E. histolytica* HM-1:IMSS human strain. Differences were also observed in the 5.8S rRNA gene and the internal transcribed spacer (ITS) regions 1 and 2, and analysis of the serine-rich protein gene from the monkey strain showed unique codon usages compared to *E. histolytica* isolated from humans. The amino acid sequences of two hexokinases and two glucose phosphate isomerases also differed from those of *E. histolytica*. Isoenzyme analyses of these enzymes in the monkey strain showed different electrophoretic mobility patterns compared with *E. histolytica* isolates. Analysis of peroxiredoxin genes indicated the presence of at least seven different types of protein, none of which were identical to proteins in *E. histolytica*. When the trophozoites from the monkey strain were inoculated into the livers of hamsters, formation of amebic abscesses was observed 7 days after the injection. These results demonstrate that the strain is genetically different from *E. histolytica* and is virulent. Revival of the name *Entamoeba nuttalli* is proposed for the organism.

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**Keywords:** *Entamoeba histolytica*; *Entamoeba nuttalli*; Rhesus monkey; rRNA gene; Virulency

### 1. Introduction

The enteric protozoan *Entamoeba histolytica* causes an estimated 50 million cases of amebic colitis and liver abscess in human, resulting in 100,000 deaths annually [1]. *Entamoeba dispar* is morphologically indistinguishable from *E. histolytica* but is nonpathogenic [2]. It has been reported that *E. histolytica/E. dispar* is commonly found in the feces of non-human primates, such as macaques and baboons [3–6]. Since *E. his-*

*tolytica* infection in non-human primates is problematic not only for animal health but also as a possible source of transmission to humans, it is important to discriminate between *E. histolytica* and *E. dispar*. Recent studies have demonstrated that *E. dispar* infection is prevalent in captive and wild non-human primates, including baboons, macaques, and chimpanzees [7–11]. We have demonstrated that the prevalence of *E. dispar*, but not *E. histolytica*, is 43% in a wild colony of *Macaca fuscata fuscata* in Japan [10], 66% in captive *Macaca* monkeys [8], and 56% in captive chimpanzees [9]. In contrast, a limited number of *E. histolytica* infections in non-human primates have been confirmed [12–14]. However, it is unknown whether *E. histolytica* isolated from non-human primates is identical to that isolated from humans.

In the present study, we analyzed several genes of an *Entamoeba* strain isolated from a rhesus monkey, *Macaca mulatta*, and compared it with a reference strain isolated from humans.

**Abbreviations:** GPI, glucose phosphate isomerase; HXK, hexokinase; ITS, internal transcribed spacer; PAS, periodic acid-Schiff; PRX, peroxiredoxin

<sup>☆</sup> **Note:** Nucleotide sequence data reported in this paper are available in the DDBJ, EMBL, and GenBank<sup>TM</sup> databases under the accession numbers AB282657 to AB282673.

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We report here that the monkey strain is virulent but genetically different from *E. histolytica*.

## 2. Materials and methods

### 2.1. Isolation and culture conditions

A fecal sample containing *Entamoeba* cysts was obtained from a rhesus monkey in Pashupati Nath Temple, Kathmandu, Nepal on June 2005. The sample was suspended in water for 24 h to remove *Blastocystis* spp. and then cultured in modified Tanabe-Chiba medium, which consists of an agar slant and an upper liquid medium in a screw-cap test tube, at 37 °C. The slant contained 1% agar in Ringer's solution supplemented with 0.1% L-asparagine, and the liquid medium contained phosphate-buffered saline (pH 7.6) supplemented with one-eighth the volume of horse serum. A microspatula of rice powder was added before use. Grown trophozoites of the *Entamoeba*, designated as the P19-061405 strain, were brought to Japan and cultured in Robinson's medium at 37 °C [15]. The trophozoites were treated with a cocktail of antibiotics and then cultured monoxenically with live *Crithidia fasciculata* in BI-S-33 medium supplemented with 15% adult bovine serum at 37 °C [16]. The monoxenically cultured trophozoites were used in most experiments in the present study. Finally, trophozoites of the strain were cultured axenically in the BI-S-33 medium and then cloned by limiting dilution, followed by examination using microscopy. Trophozoites of *E. histolytica* strains HM-1:IMSS, HK-9, Rahman, and NOT-12 were cultured axenically in BI-S-33 medium

and were used as reference strains. Trophozoites of the *E. histolytica* SAW1453 strain were cultured xenically in Robinson's medium. Trophozoites of the *E. dispar* SAW1734RclAR strain were grown monoxenically with sterilized *C. fasciculata* in YIGADHA-S medium supplemented with 15% adult bovine serum at 37 °C and were also used as a reference strain [17].

### 2.2. PCR amplification

Genomic DNA of trophozoites was isolated using a DNeasy tissue kit (Qiagen). The primers used for PCR amplification are listed in Table 1. PCR of peroxiredoxin (PRX) genes was performed to distinguish between *E. histolytica* and *E. dispar*, as previously described [18]. Amplification of the serine-rich protein gene was performed essentially as reported by Ghosh et al. [19]. Ribosomal RNA genes, hexokinase (HXK) genes, glucose phosphate isomerase (GPI) genes, and full-length PRX genes were amplified 30 cycles using *Pyrobest* DNA polymerase (Takara). The PCR conditions were as follows: denaturation at 94 °C for 15 s (195 s in cycle 1), annealing at 60 °C (for ribosomal RNA genes), or 58 °C (for other genes) for 30 s, and extension at 72 °C for 120 s (600 s in cycle 30). The sizes of PCR products were confirmed by electrophoresis on agarose gels.

### 2.3. Sequencing

PCR products of the ribosomal RNA gene and serine-rich protein gene were purified using a QIAEX II gel extraction kit

Table 1  
Primers used in this study

Gene or protein (species)	Direction: Sequence (5'–3')	GeneBank accession no.
PRX (Eh)	S (P11): GGA GGA GTA GGA AAG TTG AC AS (P12): TTC TTG CAA TTC CTG CTT CGA	D10512
PRX (Ed)	S (P13): AGG AGG AGT AGG AAA ATT AGG AS (P14): TTC TTG AAA CTC CTG TTT CTA C	D00872
Serine-rich protein (Eh)	S: GCT AGT CCT GAA AAG CTT GAA GAA GCT G AS: GGA CTT GAT GCA GCA TCA AGG T	M80910
rRNA (Eh)	S: GAA GAA GTC AAA AGT AGG AGA ATA AAG AAA GG AS: CCC TTA TTG ATA TGC TTA AGT TAA GGG AGTC	X65163
HXK (Eh)	S: ATG CAA GAA ATC ATT GAT CAA TTT AS: TTA GTG TTT ACA TGC AAC AGC A	X82197, X82198
HXK1	AS: TTA GTG TTT ACA TGC AAC AGC A	X82197
HXK2	AS: TTA TTG TTT GCA TGC AAC AGC A	X82198
GPI (Eh)	S: ATG TTA CCA ACT CTT CCT GAA T AS: TTA GTT TTT TCT CAT ATC TTT AAC A	AB217859
PRX Full (Eh)	S: ATG TCT TGC AAT CAA CAA AAA GA AS: TTA ATG TGC TGT TAA ATA TTT CTT AAT	X70996
18S rRNA (Eh)	S: GTT TTA TAC ATT TTG AAG ACT TTA TG AS: CAG ATC TAG AAA CAA TGC TTC TCT	X65163
18S rRNA (Ed)	S: ATT TTA TAC ATT TTG AAG ACT TTA CAT T AS: GAA CAA GGT AGT ATT GAT ATA CTT G	AB282661*
18S rRNA (P19)	S: TTT TAT ACA TTT TGA AGA CTT TGC ATA AS: AAG GTA ATA TTG ATA TAC TCA GAT TA	AB282657*

Eh, *E. histolytica*; Ed, *E. dispar*; P19, *Entamoeba* sp. P19-061405 strain; S, Sense; AS, Anti-sense.

\* Present study.

(Qiagen) and subjected to direct sequencing. Amplified genes for HXK, GPI, and PRX were cloned using a Zero Blunt TOPO PCR cloning kit for sequencing (Invitrogen). Eight clones each from the *HXK* and *GPI* genes and 12 clones from *PRX* genes were subjected to sequencing, which was performed with a BigDye Terminator v3.1 cycle sequencing kit (Applied Biosystems). The reactions were run on an ABI Prism 3100 genetic analyzer (Applied Biosystems). Sequence data were analyzed using Genetyx-Mac ver. 11 (Genetyx).

#### 2.4. Sandwich ELISA

Approximately  $10^3$  trophozoites cultured monoxenically with *C. fasciculata* in BI-S-33 medium were analyzed using an *E. histolytica* antigen detection kit (*E. HISTOLYTICA II*, Tech-Lab). The protocol for stool samples was also used for cultured samples.

#### 2.5. Indirect immunofluorescence microscopy

The reactivity of the *E. histolytica*-specific monoclonal antibody 4G6 against PRX was examined by indirect immunofluorescence microscopy, as described previously, except that monoxenic trophozoites were fixed with 4% paraformaldehyde [12,20].

#### 2.6. Zymodeme analysis

Isoenzyme analysis of *Entamoeba* trophozoites in starch gel was performed according to the method of Sargeant [21].

#### 2.7. Hepatic inoculation

Five male Syrian hamsters of 8 weeks of age were used in the study. Three hamsters were anesthetized by intraperitoneal injection of pentobarbital and then  $3 \times 10^5$  trophozoites cultured monoxenically with *C. fasciculata* were inoculated into the left lobe of the liver. Two control hamsters received inoculation of live *C. fasciculata*. The hamsters were sacrificed 7 days after inoculation to examine the formation of amebic liver abscesses. Liver tissues, including abscesses, were fixed with 4% paraformaldehyde in phosphate buffer and then embedded in paraffin. Thin sections were stained with hematoxylin and eosin, and with periodic acid-Schiff (PAS) reagent.

### 3. Results

#### 3.1. Identification of the P19-061405 strain by conventional methods

Cysts of *Entamoeba* in fecal samples were morphologically identified as *E. histolytica/E. dispar*. After the sample was transferred to culture, the trophozoites grew well in Tanabe-Chiba medium and Robinson's medium under xenic conditions. PCR amplification analysis of *PRX* genes demonstrated that the isolate P19-061405 was *E. histolytica* (Fig. 1). The trophozoites were also successfully cultured in BI-S-33 medium with *C.*

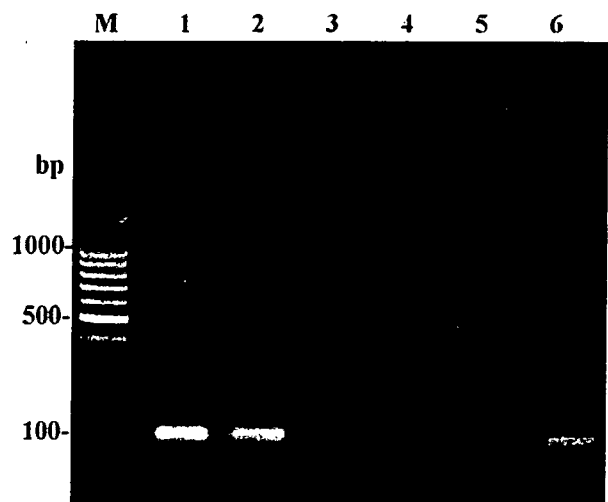


Fig. 1. PCR amplification of peroxiredoxin genes from trophozoites of the P19-061405 strain to distinguish between *E. histolytica* and *E. dispar*. Two sets of specific primers were used: P11 and P12 for *E. histolytica* (lanes 1–3) and P13 and P14 for *E. dispar* (lanes 4–6) [18]. Templates are genomic DNA from P19-061405 (lanes 1 and 4), *E. histolytica* HM-1:IMSS (lanes 2 and 5), and *E. dispar* SAW1734RclAR (lanes 3 and 6). M, DNA size marker (100 bp ladder).

*fasciculata* at 37 °C. No morphological differences were evident under light microscopy in comparison with human isolates of *E. histolytica*. Analysis of cultured trophozoites with the *E. histolytica* antigen detection kit indicated a positive optical density. Trophozoites of the isolate were also reactive with the *E. histolytica*-specific monoclonal antibody 4G6.

#### 3.2. Analysis of the ribosomal RNA gene

A region of ribosomal RNA genes of about 2.4 kb was amplified by PCR and sequenced directly. Sequence analysis of the 18S ribosomal RNA gene of the P19-061405 strain showed an identical size but a 0.8% (15/1945) difference in sequence, compared with the reported *E. histolytica* HM-1:IMSS strain (Fig. 2). On the other hand, the difference between P19-061405 and *E. dispar* SAW1734RclAR was 1.2% in the overlapping region. This difference is smaller than that the 1.5% difference between *E. histolytica* HM-1:IMSS and *E. dispar* SAW1734RclAR, indicating that the monkey strain is phylogenetically located between *E. histolytica* and *E. dispar*. Differences between the monkey strain and the human reference *E. histolytica* strain were also compared for the 5.8S ribosomal RNA gene and the internal transcribed spacer (ITS) 1 and 2 regions. One- and two-nucleotide differences were found in the 5.8S rRNA gene and ITS1 region, respectively. In the ITS2 region, a one-nucleotide insertion and a deletion of 5 nucleotides were observed in the monkey strain, in addition to substitution of 6 nucleotides. In other human *E. histolytica* strains, HK-9, Rahman, and NOT-12, no sequence differences were observed in the 2.4 kb region in comparison with the HM-1:IMSS strain. To confirm the sequence differences of the monkey strain from *E. histolytica* HM-1:IMSS and *E. dispar* SAW1734RclAR, PCR amplification of ribosomal RNA genes was performed using primers specific for each strain (Fig. 3). PCR products were detected only with

P19-18S	1	TATCGGGTGGATGCCGGCCAGTATATATGCGTGGAGGTAAAGATTAAGCCATGATGCGTAAAGTAAGAAAGCCAGTGGATGAAAGTGGCGGGGGGGCCAT	100
HM1-18S	1	TATCGGGTGGATGCCGGCCAGTATATATGCGTGGAGGTAAAGATTAAGCCATGATGCGTAAAGTAAGAAAGCCAGTGGATGAAAGTGGCGGGGGGGCCAT	100
Ed-18S	1	TATCGGGTGGATGCCGGCCAGTATATATGCGTGGAGGTAAAGATTAAGCCATGATGCGTAAAGTAAGAAAGCCAGTGGATGAAAGTGGCGGGGGGGCCAT	100
P19-18S	101	TATAACAGTAAATAGTTCCTGCTGGGTAGTAAATAATTAAGGATGATGCTGTAAGGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGAT	200
HM1-18S	101	TATAACAGTAAATAGTTCCTGCTGGGTAGTAAATAATTAAGGATGATGCTGTAAGGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGAT	200
Ed-18S	101	TATAACAGTAAATAGTTCCTGCTGGGTAGTAAATAATTAAGGATGATGCTGTAAGGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGAT	200
P19-18S	201	GCGAATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGAT	299
HM1-18S	201	GCGAATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGAT	299
Ed-18S	201	GCGAATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGAT	300
P19-18S	300	TTCTGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGAT	399
HM1-18S	300	TTCTGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGAT	399
Ed-18S	301	TTCTGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGAT	400
P19-18S	400	CTACGACCTGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGAT	499
HM1-18S	400	CTACGACCTGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGAT	499
Ed-18S	401	CTACGACCTGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGAT	500
P19-18S	500	TGAAGGAAATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGAT	599
HM1-18S	500	TGAAGGAAATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGAT	599
Ed-18S	501	TGAAGGAAATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGAT	600
P19-18S	600	ATCTGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGAT	698
HM1-18S	600	ATCTGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGAT	698
Ed-18S	601	ATCTGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGAT	700
P19-18S	699	TCAAAAGAAATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGAT	798
HM1-18S	699	TCAAAAGAAATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGAT	798
Ed-18S	701	TCAAAAGAAATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGAT	800
P19-18S	799	CAATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGAT	898
HM1-18S	799	CAATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGAT	898
Ed-18S	801	CAATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGAT	900
P19-18S	899	TAAAGAAATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGAT	998
HM1-18S	899	TAAAGAAATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGAT	998
Ed-18S	901	TAAAGAAATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGAT	1000
P19-18S	999	AACTGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGAT	1098
HM1-18S	999	AACTGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGAT	1098
Ed-18S	1001	AACTGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGAT	1100
P19-18S	1099	GAATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGAT	1198
HM1-18S	1099	GAATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGAT	1198
Ed-18S	1101	GAATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGAT	1200
P19-18S	1199	TGCGGCTGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGAT	1298
HM1-18S	1199	TGCGGCTGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGAT	1298
Ed-18S	1201	TGCGGCTGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGAT	1300
P19-18S	1299	TAGGCGGCTGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGAT	1398
HM1-18S	1299	TAGGCGGCTGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGAT	1398
Ed-18S	1301	TAGGCGGCTGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGAT	1400
P19-18S	1399	TGGTGAAGAAATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGAT	1497
HM1-18S	1399	TGGTGAAGAAATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGAT	1497
Ed-18S	1401	TGGTGAAGAAATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGAT	1500
P19-18S	1498	CGTGGGAAATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGAT	1597
HM1-18S	1498	CGTGGGAAATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGAT	1597
Ed-18S	1501	CGTGGGAAATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGAT	1600
P19-18S	1598	CTATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGAT	1697
HM1-18S	1598	CTATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGAT	1697
Ed-18S	1601	CTATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGAT	1700
P19-18S	1698	ACAAGCAGGAAATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGAT	1797
HM1-18S	1698	ACAAGCAGGAAATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGAT	1797
Ed-18S	1701	ACAAGCAGGAAATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGAT	1800
P19-18S	1798	TATAGGCTGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGAT	1897
HM1-18S	1798	TATAGGCTGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGAT	1897
Ed-18S	1801	TATAGGCTGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGAT	1900
P19-18S	1898	GGAGAAGCTGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGAT	1945
HM1-18S	1898	GGAGAAGCTGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGAT	1945
Ed-18S	1901	GGAGAAGCTGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGATGAT	1948

Fig. 2. Comparison of 18S ribosomal RNA genes from P19-061405 (top line), E. histolytica HM-1:IMSS (middle line, GenBank accession no. X65163), and E. dispar SAW1734RcIAR (bottom line). Identical and conserved nucleotide sequences are highlighted in black and gray, respectively.

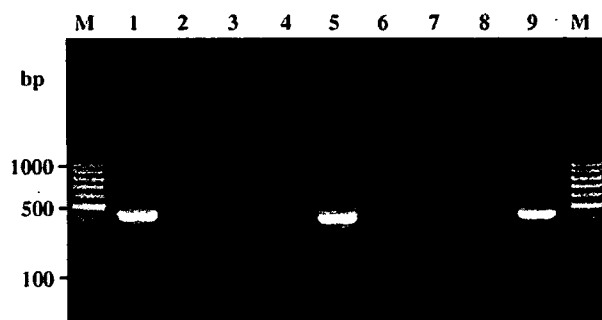


Fig. 3. PCR amplification of rRNA genes from trophozoites of P19-061405 strain, *E. histolytica*, and *E. dispar*. Three sets of primers specific for the P19-061405 strain (lanes 1–3), *E. histolytica* (lanes 4–6), and *E. dispar* (lanes 7–9) were used. Templates are genomic DNA from P19-061405 (lanes 1, 4, and 7), *E. histolytica* HM-1:IMSS (lanes 2, 5, and 8), and *E. dispar* SAW1734RclAR (lanes 3, 6, and 9). M, DNA size marker (100 bp ladder).

specific primers, indicating that the sequences of ribosomal RNA genes were clearly different among the three strains and that no contamination of *E. histolytica*/*E. dispar* was present in the monkey strain. The sequence of the 2.4-kb region in the P19-061405 strain was also determined in a clone cultured axenically and no difference was found compared to before cloning.

### 3.3. Analysis of the serine-rich protein gene

The serine-rich surface protein gene of the P19-061405 strain was amplified and sequenced directly. Repeat patterns of motifs of 3, 4, 8, and 9 amino acids differed from those in reported human isolates [19,22,23]. In the monkey strain, the motif EKASSSDKS is present, compared to EKASSSDNS in human isolates. In the DNKP motif, an AAC codon is used for Asn, whereas in human isolates this codon is AAT. Similarly, the codon for the first serine in the EASSTNKP and EASSTSNS motifs is AGT in the monkey strain and AGC in human isolates.

### 3.4. Analyses of hexokinase genes

Two *HXK* genes of the P19-061405 strain amplified from genomic DNA were cloned and sequenced. The calculated molecular mass and theoretical *pI* were 4.98 kDa and 5.38 in *HXK* 1, and 4.94 kDa and 4.99 in *HXK* 2. The deduced amino acid sequences were compared with those of *E. histolytica* and *E. dispar*. Differences between the P19-061405 strain and *E. histolytica* were observed in 3 and 4 of 445 amino acids in *HXK* 1 and 2, respectively. On the other hand, differences between the monkey strain and *E. dispar* were found for 13 and 15 of the 445 amino acids in *HXK* 1 and 2, respectively. The *pI* of *HXK* 1 from the monkey strain differed from that of *E. histolytica* *HXK* 1, whereas the *pI* of *HXK* 2 was identical in the two strains.

### 3.5. Analyses of glucose phosphate isomerase genes

Two *GPI* genes from the P19-061405 strain encoded proteins of 546 amino acids with calculated molecular masses of 6.14 kDa. Lys<sup>362</sup> in the *E. histolytica* *GPIs* was changed to Glu in the two *GPIs* of the monkey strain. Two additional differences

were found: Ile<sup>232</sup> and Val<sup>330</sup> were present in one *GPI* from the monkey strain, whereas Val and Ala, respectively, were present at these positions in the other *GPIs*. The theoretical *pI* of the two *GPIs* from the monkey strain was 6.6, which differed from those of the three *E. histolytica* *GPIs*.

### 3.6. Analyses of peroxiredoxin genes

Seven different sequences of *PRX* genes were detected in 12 clones from the P19-061405 strain. Using the deduced amino acids, 7 different *PRX* sequences were identified: 5 had 233 amino acids, 1 had 237 amino acids, and 1 had 229 amino acids. However, none of these sequences were identical to the 6 *PRX* sequences from the *E. histolytica* HM-1:IMSS strain in the genome database (<http://www.tigr.org/tdb/>). In the P19-061405 strain, Val<sup>156</sup> in *E. histolytica* *PRXs* is replaced with Phe in 5 of the 7 sequences, and Ala<sup>161</sup> in *E. histolytica* *PRXs* is replaced with Ser in 6 sequences. In each *PRX* in the monkey strain, at least one of these two positions was different to *E. histolytica*. In addition, several unique amino acids were found in specific positions in some *PRX* clones from the P19-061405 strain: Arg<sup>130</sup>, Ile<sup>179</sup>, Glu<sup>202</sup>, Glu<sup>217</sup>, and Asn<sup>224</sup>.

### 3.7. Analyses of isoenzyme patterns

Electrophoretic patterns of four enzymes from the P19-061405 strain are shown in Fig. 4. In *HXK*, the slower running band in the monkey strain was located between those of *E. histolytica* and *E. dispar*, whereas the mobility of the faster running band was similar to that for *HXK* from *E. histolytica*. For *GPI*, only a  $\gamma$  band was observed. Therefore, the band patterns for *HXK* and *GPI* appear to be novel in the P19-061405 strain. The mobility of the L-malate:NADP<sup>+</sup> oxidoreductase band was same as that for *E. histolytica*/*E. dispar*, and the mobility of the phosphoglucosyltransferase band was the same as that for *E. dispar*.

### 3.8. Virulence of the monkey strain in hamster

When trophozoites of P19-061405 cultured monoxenically with *C. fasciculata* were intrahepatically inoculated into livers of three hamsters, formation of abscesses was observed in all hamsters (Fig. 5). Direct light microscopic examination of the abscess detected moving trophozoites. The presence of many trophozoites was demonstrated in the peripheral region of abscesses in the livers by PAS staining of thin sections (Fig. 6). In two control hamsters, no abscess was detected in livers inoculated with *C. fasciculata* only.

## 4. Discussion

The present study demonstrated that the monkey strain P19-061405 is similar to *E. histolytica*, but the sequences of ribosomal RNA genes were different from those in *E. histolytica* isolated from humans. Differences in 5.8S ribosomal RNA genes and the ITS 1 and 2 regions of *E. histolytica* have been reported among strains HM-1:IMSS, Rahman, and HK-9 [24], but it has

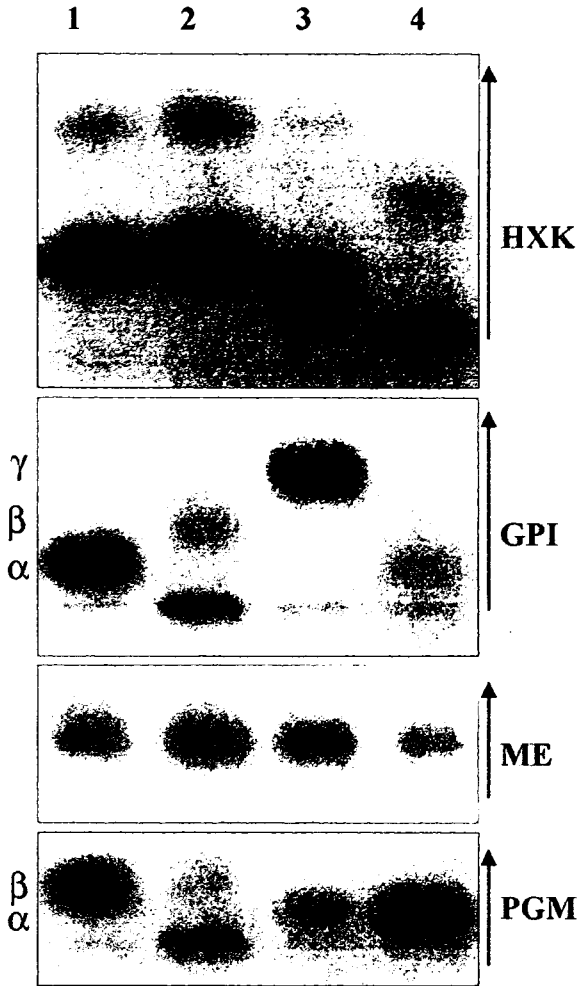


Fig. 4. Isoenzyme patterns of HXK, GPI, L-malate:NADP<sup>+</sup> oxidoreductase (ME), and phosphoglucutase (PGM) from axenically cultured trophozoites of the P19-061405 strain (lane 3). Lane 1, *E. histolytica* HM-1:IMSS strain (Zymodeme II); lane 2, *E. histolytica* SAW1453 strain (Zymodeme XIV); and lane 4, *E. dispar* SAW1734RclAR strain (Zymodeme I). Arrows indicate the direction of migration.

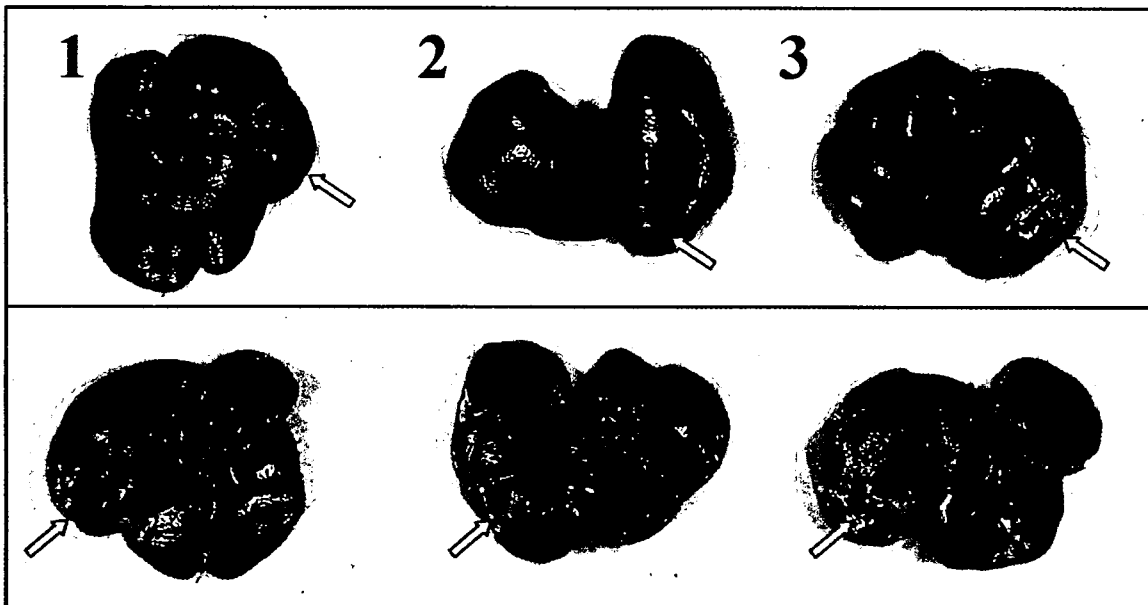


Fig. 5. Livers from 3 hamsters inoculated with trophozoites of the P19-061405 strain 7 days before analysis. Surface (above) and back (below) views are shown. Arrows indicate the location of amebic liver abscesses.



Fig. 6. PAS-stained section of a liver from a hamster that received intrahepatic inoculation of P19-061405 trophozoites. Abscesses are apparent in the bottom half of the photograph, and many PAS-positive trophozoites (arrows) are present in the peripheral region of the abscess. Bar indicates 50 μm.

also been shown that these sequences are identical in 10 strains of *E. histolytica* [25]. In this study, sequence differences in ribosomal RNA genes were not found among human-derived strains of *E. histolytica*, HM-1:IMSS, HK-9, Rahman, and NOT-12. It has also been reported that the sequence of the 18S ribosomal RNA gene is identical in 14 isolates of *E. histolytica* [26]. Therefore, sequences of ribosomal RNA genes seem to be highly conserved in human isolates of *E. histolytica*. The possibility that the sequence difference between P19-061405 and *E. histolytica* is due to PCR or sequencing artifacts, or due to the presence of mixed populations, was ruled out by sequencing and PCR amplification using specific primers, as shown in Fig. 3. The extent of the difference in the 18S ribosomal RNA gene between P19-061405 and *E. histolytica* is only about half that between the genes in *E. histolytica* and *E. dispar*, but this difference appears

to be significant for discrimination of the monkey strain from *E. histolytica*.

Genetic differences between the P19-061405 strain and *E. histolytica* were also demonstrated in related proteins using zymodeme analysis. In this analysis, HXK is a key enzyme for discrimination between *E. histolytica* and *E. dispar* [27]. The primary structures of two proteins, HXK 1 and 2, in the monkey strain are clearly different from those of *E. histolytica* and *E. dispar* [28]. Recently, it has also been demonstrated that zymodemes of *E. histolytica*, Z-II, -II $\alpha$ -, -XIV, and -XIX, are expressed by six alleles of *GPI* genes [29]. Two *GPI* genes in the monkey strain encoded proteins that differed from three *GPI* proteins in *E. histolytica*. Indeed, zymodeme analysis of the monkey strain demonstrated unique patterns for HXK and *GPI*. The position of the slowly migrating band in HXK is explained by the different *pI* of HXK 1 from the monkey strain, compared to that of *E. histolytica* HXK 1. The appearance of a  $\gamma$  band for *GPI* is also reasonable, since the two *GPI*s from the monkey strain had identical *pI*s that differed from those of the three *E. histolytica* *GPI*s. These differences in the monkey strain do not seem to be intraspecific variations of *E. histolytica* isolates.

We have reported that PCR analysis of *PRX* genes is useful for discrimination between *E. histolytica* and *E. dispar*, and we have also detected *E. histolytica* in clinical samples such as abscesses [18,30–32]. Although the PCR analysis was unable to discriminate between the monkey strain and *E. histolytica*, the protocol still seems to be valuable because the monkey strain was virulent. The small sequence differences in *PRX* genes between the monkey and human isolates allow design of primers specific for the monkey strain. Comparison of the sequences of *PRX* genes from *E. histolytica*, *E. dispar*, and *E. moshkovskii* indicate large differences in the N-terminus of the protein: an insertion in *E. dispar* and a deletion in *E. moshkovskii* [33,34]. However, size differences of *PRX* were not detected between the monkey strain and *E. histolytica*. Therefore, the common primary structure of *PRX* in these two strains may suggest an important role of the protein in pathogenesis, in addition to a housekeeping role [35,36].

We have previously isolated *E. histolytica* from a Japanese macaque, *Macaca fuscata fuscata* [12]. However, no significant differences at the genetic level were detected between the isolate and the *E. histolytica* reference strain in preliminary experiments (Tachibana et al., unpublished data). Recently, an *E. histolytica*-like strain has also been isolated from a cynomolgus monkey, *Macaca fascicularis*, which was imported into Japan from China for research use [14]. Sequence analysis of the serine-rich protein gene in the strain revealed unique codon usages in comparison with *E. histolytica* human isolates (Takano et al., unpublished data). It has been shown that there are differences in codon usage between *E. histolytica* and *E. dispar* and also between high expression and low expression proteins of *E. histolytica* [37–39]. Since unique codon usage was also observed in the present study, it may be a common feature in monkey strains and strongly indicates that *E. histolytica*-like strains from monkeys are phylogenetically different to human isolates.

The host rhesus monkey that delivered the *Entamoeba* cysts seemed to be asymptomatic, since the stool was not loose or bloody. However, the monkey strain was virulent when trophozoites were inoculated into hamsters. Therefore, we cannot exclude the possibility that the monkey strain is infective to humans and could cause human amebiasis. It would be interesting to investigate whether the monkey strain is dominant in monkey colonies, to determine if an infection with *E. histolytica* identical to human isolates exists in the colony, and to find evidence for human cases of natural infection with the monkey strain. Epidemiological studies are required to answer these questions.

Invasive amebiasis diagnosed by histopathology has been reported in captive and in wild-trapped non-human primates [40–43]. The present study suggests that we cannot exclude the possibility that these cases were due to the P19-061405 type of *Entamoeba*, but misdiagnosed as *E. histolytica*. In older literature, the name *Entamoeba nuttalli* was proposed for a *E. histolytica*-like species found in a liver abscess in a *Macacus pileatus* monkey in Colombo, Ceylon [44]. Furthermore, several *E. histolytica*-like amebas in monkeys have been described as *E. duboscqi*, *E. chattoni*, *E. ateles*, and *E. cynomolgi*. Except for *E. chattoni*, which is single nucleate, these species may really be *E. nuttalli*, which has been thought of as synonymous to *E. histolytica* [45,46].

In conclusion, the *E. histolytica*-like strain isolated from rhesus monkey is virulent, but genetically different from *E. histolytica* human isolates. We propose that the organism be separated from *E. histolytica* by revival of the name *E. nuttalli* Castellani [44].

#### Acknowledgements

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#### References

- [1] Walsh JA. Problems in recognition and diagnosis of amebiasis: estimation of the global magnitude of morbidity and mortality. *Rev Infect Dis* 1986;8:228–38.
- [2] Diamond LS, Clark CG. A redescription of *Entamoeba histolytica* Schaudinn, 1903 (Emended Walker, 1911) separating it from *Entamoeba dispar* Brumpt 1925. *J Eukaryot Microbiol* 1993;40:340–4.
- [3] Ghandour AM, Zahid NZ, Banaja AA, Kamal KB, Bouq AI. Zoonotic intestinal parasites of hamadryas baboons *Papio hamadryas* in the western and northern regions of Saudi Arabia. *J Trop Med Hyg* 1995;98:431–9.
- [4] Eberhard ML. Intestinal parasitism in an outdoor breeding colony of *Macaca mulatta*. *Lab Anim Sci* 1981;31:282–5.
- [5] Sano M, Kino H, de Guzman TS, et al. Studies on the examination of imported laboratory monkey *Macaca fascicularis* for *E. histolytica* and other intestinal parasites. *Int J Zoonoses* 1980;7:34–9.
- [6] Legesse M, Erko B. Zoonotic intestinal parasites in *Papio anubis* (baboon) and *Cercopithecus aethiops* (vervet) from four localities in Ethiopia. *Acta Trop* 2004;90:231–6.
- [7] Smith JM, Meerovitch E. Primates as a source of *Entamoeba histolytica*, their zymodeme status and zoonotic potential. *J Parasitol* 1985;71:751–6.



- [8] Tachibana H, Cheng XJ, Kobayashi S, Matsubayashi N, Gotoh S, Matsubayashi K. High prevalence of infection with *Entamoeba dispar*, but not *E. histolytica*, in captive macaques. *Parasitol Res* 2001;87:14–7.
- [9] Tachibana H, Cheng XJ, Kobayashi S, Fujita Y, Uono T. *Entamoeba dispar*, but not *E. histolytica*, detected in a colony of chimpanzees in Japan. *Parasitol Res* 2000;86:537–41.
- [10] Rivera WL, Kanbara H. Detection of *Entamoeba dispar* DNA in macaque feces by polymerase chain reaction. *Parasitol Res* 1999;85:493–5.
- [11] Jackson TF, Sargeant PG, Visser PS, Gathiram V, Suparsad S, Anderson CB. *Entamoeba histolytica*: naturally occurring infections in baboons. *Arch Invest Med (Mex)* 1990;21(Suppl.):153–6.
- [12] Tachibana H, Kobayashi S, Kato Y, Nagakura K, Kaneda Y, Takeuchi T. Identification of a pathogenic isolate-specific 30000-Mr antigen of *Entamoeba histolytica* by using a monoclonal antibody. *Infect Immun* 1990;58:955–60.
- [13] Verweij JJ, Vermeer J, Brienen EA, et al. *Entamoeba histolytica* infections in captive primates. *Parasitol Res* 2003;90:100–3.
- [14] Takano J, Narita T, Tachibana H, et al. *Entamoeba histolytica* and *Entamoeba dispar* infections in cynomolgus monkeys imported into Japan for research. *Parasitol Res* 2005;97:255–7.
- [15] Robinson GL. The laboratory diagnosis of human parasitic amoebae. *Trans R Soc Trop Med Hyg* 1968;62:285–94.
- [16] Diamond LS, Harlow DR, Cunnick CC. A new medium for the axenic cultivation of *Entamoeba histolytica* and other *Entamoeba*. *Trans R Soc Trop Med Hyg* 1978;72:431–2.
- [17] Kobayashi S, Imai E, Haghghi A, Khalifa SA, Tachibana H, Takeuchi T. Axenic cultivation of *Entamoeba dispar* in newly designed yeast extract-iron-gluconic acid-dihydroxyacetone-serum medium. *J Parasitol* 2005;91:1–4.
- [18] Tachibana H, Kobayashi S, Takekoshi M, Ihara S. Distinguishing pathogenic isolates of *Entamoeba histolytica* by polymerase chain reaction. *J Infect Dis* 1991;164:825–6.
- [19] Ghosh S, Frisardi M, Ramirez-Avila L, et al. Molecular epidemiology of *Entamoeba* spp. evidence of a bottleneck (Demographic sweep) and transcontinental spread of diploid parasites. *J Clin Microbiol* 2000;38:3815–21.
- [20] Tachibana H, Kobayashi S, Nagakura K, Kaneda Y, Takeuchi T. Asymptomatic cyst passers of *Entamoeba histolytica* but not *Entamoeba dispar* in institutions for the mentally retarded in Japan. *Parasitol Int* 2000;49:31–5.
- [21] Sargeant PG. Zymodemes of *Entamoeba histolytica*. In: Ravdin JJ, editor. *Amebiasis: human infection by Entamoeba histolytica*. New York: John Wiley & Sons; 1988. p. 370–87.
- [22] Haghghi A, Kobayashi S, Takeuchi T, Masuda G, Nozaki T. Remarkable genetic polymorphism among *Entamoeba histolytica* isolates from a limited geographic area. *J Clin Microbiol* 2002;40:4081–90.
- [23] Haghghi A, Kobayashi S, Takeuchi T, Thammapalerd N, Nozaki T. Geographic diversity among genotypes of *Entamoeba histolytica* field isolates. *J Clin Microbiol* 2003;41:3748–56.
- [24] Som I, Azam A, Bhattacharya A, Bhattacharya S. Inter- and intra-strain variation in the 5.8S ribosomal RNA and internal transcribed spacer sequences of *Entamoeba histolytica* and comparison with *Entamoeba dispar*, *Entamoeba moshkovskii* and *Entamoeba invadens*. *Int J Parasitol* 2000;30:723–8.
- [25] Newton-Sanchez OA, Sturm-Ramirez K, Romero-Zamora JL, Santos-Preciado JJ, Samuelson J. High rate of occult infection with *Entamoeba histolytica* among non-dysenteric Mexican children. *Arch Med Res* 1997;28:311–3.
- [26] Bhattacharya D, Haque R, Singh U. Coding and noncoding genomic regions of *Entamoeba histolytica* have significantly different rates of sequence polymorphisms: implications for epidemiological studies. *J Clin Microbiol* 2005;43:4815–9.
- [27] Sargeant PG. Zymodemes of *Entamoeba histolytica*. *Parasitol Today* 1987;3:158.
- [28] Ortner S, Clark CG, Binder M, Scheiner O, Wiedermann G, Duchene M. Molecular biology of the hexokinase isoenzyme pattern that distinguishes pathogenic *Entamoeba histolytica* from nonpathogenic *Entamoeba dispar*. *Mol Biochem Parasitol* 1997;86:85–94.
- [29] Razmjou E, Haghghi A, Rezaian M, Kobayashi S, Nozaki T. Genetic diversity of glucose phosphate isomerase from *Entamoeba histolytica*. *Parasitol Int* 2006;55:307–11.
- [30] Ohnishi K, Murata M, Kojima H, Takemura N, Tsuchida T, Tachibana H. Brain abscess due to infection with *Entamoeba histolytica*. *Am J Trop Med Hyg* 1994;51:180–2.
- [31] Rivera WL, Tachibana H, Kanbara H. Field study on the distribution of *Entamoeba histolytica* and *Entamoeba dispar* in the northern Philippines as detected by the polymerase chain reaction. *Am J Trop Med Hyg* 1998;59:916–21.
- [32] Tachibana H, Kobayashi S, Okuzawa E, Masuda G. Detection of pathogenic *Entamoeba histolytica* DNA in liver abscess fluid by polymerase chain reaction. *Int J Parasitol* 1992;22:1193–6.
- [33] Tachibana H, Cheng XJ. *Entamoeba dispar*: cloning and characterization of peroxiredoxin genes. *Exp Parasitol* 2000;94:51–5.
- [34] Cheng XJ, Yoshihara E, Takeuchi T, Tachibana H. Molecular characterization of peroxiredoxin from *Entamoeba moshkovskii* and a comparison with *Entamoeba histolytica*. *Mol Biochem Parasitol* 2004;138:195–203.
- [35] Choi MH, Sajed D, Poole L, et al. An unusual surface peroxiredoxin protects invasive *Entamoeba histolytica* from oxidant attack. *Mol Biochem Parasitol* 2005;143:80–9.
- [36] Davis PH, Zhang X, Guo J, Townsend RR, Stanley Jr SL. Comparative proteomic analysis of two *Entamoeba histolytica* strains with different virulence phenotypes identifies peroxiredoxin as an important component of amoebic virulence. *Mol Microbiol* 2006;61:1523–32.
- [37] Ghosh TC, Gupta SK, Majumdar S. Studies on codon usage in *Entamoeba histolytica*. *Int J Parasitol* 2000;30:715–22.
- [38] Tannich E, Horstmann RD. Codon usage in pathogenic *Entamoeba histolytica*. *J Mol Evol* 1992;34:272–3.
- [39] Nozaki T, Asai T, Takeuchi T. Codon usage in *Entamoeba histolytica*, *E. dispar* and *E. invadens*. *Parasitol Int* 1997;46:105–9.
- [40] Loomis MR, Britt Jr JO, Gendron AP, Holshuh HJ, Howard EB. Hepatic and gastric amebiasis in black and white colobus monkeys. *J Am Vet Med Assoc* 1983;183:1188–91.
- [41] Amyx HL, Asher DM, Nash TE, Gibbs Jr CJ, Gajdusek DC. Hepatic amebiasis in spider monkeys. *Am J Trop Med Hyg* 1978;27:888–91.
- [42] Palmieri JR, Dalgard DW, Connor DH. Gastric amebiasis in a silvered leaf monkey. *J Am Vet Med Assoc* 1984;185:1374–5.
- [43] Beaver PC, Blanchard JL, Seibold HR. Invasive amebiasis in naturally infected New World and Old World monkeys with and without clinical disease. *Am J Trop Med Hyg* 1988;39:343–52.
- [44] Castellani A. Note on a liver abscess of amoebic origin in a monkey. *Parasitology* 1908;1:101–2.
- [45] Neal RA. Experimental studies on *Entamoeba* with reference to speciation. *Adv Parasitol* 1966;4:1–51.
- [46] Hegner R, Schumaker E. Some intestinal amoebae and flagellates from the chimpanzee, three-toed sloth, sheep and guinea-pig. *J Parasitol* 1928;15:31–7.

## Comparison of *Entamoeba histolytica* DNA isolated from a cynomolgus monkey with human isolates

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**Abstract** Three protein-coding loci in DNA of an *Entamoeba histolytica* strain (EHMfas1) isolated from cynomolgus monkey (*Macaca fascicularis*) were sequenced; these loci corresponded to the genes for chitinase, the serine-rich *E. histolytica* protein (SREHP), and the 16 S-like small subunit ribosomal RNA (16S-like SSUrRNA). The nucleotide and deduced amino-acid sequences of chitinase and SREHP were compared with sequences from human isolates. EHMfas1 had several specific mutations in units in the polymorphic regions of the chitinase and SREHP loci, with some repetition of these mutated units. The sequence of the 16S-like SSUrRNA gene (16S-like SSUrDNA) was compared with other *Entamoeba* species. In phylogenetic analysis, EHMfas1 was not categorized in the *E. histolytica* cluster but between *E. histolytica* and *E. dispar*. To our knowledge, this is the first molecular characterization of *E. histolytica* isolated from cynomolgus monkey, and our results indicate that EHMfas1 may be a subspecies of *E. histolytica* that infects cynomolgus monkey.

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### Introduction

Amoebiasis is caused by infection with *Entamoeba histolytica* and is one of the most important parasitic diseases in humans. In nonhuman primates, amoebiasis has been reported in captive and in wild-trapped nonhuman primates (Amyx et al. 1978; Beaver et al. 1988), but most of these reports did not distinguish *E. histolytica* from *E. dispar*, which is morphologically similar to *E. histolytica* but nonpathogenic. One case of *E. histolytica* infection in Japanese macaque (*Macaca fuscata*) was determined by isoenzyme analysis (Tachibana et al. 1990), and PCR tests have been used to identify six cases of infections in three species of Old World monkeys and three species of New World monkeys (Verweij et al. 2003) and, in one case, in cynomolgus monkey (*M. fascicularis*; Takano et al. 2005). However, it is unclear whether the *E. histolytica* strains in nonhuman primates originated from humans or are species-specific strains.

The genes for chitinase (de la Vega et al. 1997; Ghosh et al. 2000; Haghighi et al. 2002, 2003) and serine-rich *E. histolytica* protein (SREHP; also known as K2; Li et al. 1992; Clark and Diamond 1993; Kohler and Tannich 1993; Stanley et al. 1990; Ayeh-Kumi et al. 2001; Haghighi et al. 2002, 2003) have highly polymorphic DNA loci, including the protein-coding sequences. These polymorphic loci have been used to study the molecular epidemiology and geographic diversity within human isolates of *E. histolytica*. Genes for the 16 S-like small subunit ribosomal RNA (16S-like SSUrDNA; Medlin et al. 1988) have been used for identification of species (Clark and Diamond 1991; Novati et al. 1996), and phylogenetic analysis of the genus *Entamoeba* has been performed using riboprinting (Clark and Diamond 1997) and nucleotide sequencing (Silberman et al. 1999; Clark et al. 2006).

In this study, we determined the DNA sequences of the chitinase and SREHP loci of the *E. histolytica* strain EHMfas1 and compared these sequences with those from human isolates. We also examined the 16S-like SSUrDNA gene sequence to compare EHMfas1 with other *Entamoeba* species.

## Materials and methods

### *E. histolytica* from cynomolgus monkey

The *E. histolytica* strain EHMfas1 was isolated from a healthy cynomolgus monkey imported into Japan from China (Takano et al. 2005).

### DNA preparation and sequencing

Total genomic DNA from trophozoites was extracted using a QIAamp DNA Stool Mini Kit (Qiagen) according to the manufacturer's directions. Chitinase and SREHP were amplified by PCR using previously described primer sets: 5'-GGA ACA CCA GGT AAA TGT ATA-3' and 5'-TCT GTA TTG TGC CCA ATT-3' for the chitinase forward and reverse primers, respectively, and 5'-GCT AGT CCT GAA AAG CTT GAA GAA GCT G-3' and 5'-GGA CTT GAT GCA GCA TCA AGG T-3' for the SREHP forward and reverse primers, respectively (Ghosh et al. 2000). Polymerase chain reaction (PCR) was carried out in a 50- $\mu$ l reaction mixture containing 2  $\mu$ l of extracted DNA and 0.1  $\mu$ g of bovine serum albumin per microliter using Takara Ex Taq (Takara). A total of 35 cycles of PCR were performed as follows: denaturation at 94°C for 30 s, annealing at 54°C for 30 s, and extension at 72°C for 45 s. An initial denaturation step at 94°C for 5 min and a final extension step at 72°C for 7 min were also performed.

16S-like SSUrDNA was also amplified using previously described primer sets (RD5 and RD3; Clark and Diamond 1997), with some modification, and newly designed internal primer sets to obtain accurate nucleotide sequencing: RD5, 5'-ATC TGG TTG ATC CTG CCA GT-3' and 1r, 5'-TCG TCA CTA CCT CTT CAA TTC G-3'; 2f, 5'-CAC TTC TAA GGA AGG CAG CAG-3' and 2r, 5'-AGC GAT CAT GGA TTT TCA CC-3'; 3f, 5'-AAA AGG AAC AAT TGG GGT GA-3' and 3r, 5'-CCA TGC ACC ACT ACC CAA TA-3'; 4f, 5'-TGG TCA CAA GGC TGA AAC TT-3' and 4r, 5'-CCA AGA TGT CTA AGG GCA TCA-3'; 5f, 5'-GGG AAA AAG AAA AAG GAA GCA-3' and RD3, 5'-ATC CTT CCG CAG GTT CAC CT-3'). A total of 30 cycles of PCR were performed as follows: denaturation at 94°C for 1 min, annealing at 58°C for 30 s, and extension at 72°C for 1 min. An initial denaturation step at 94°C for 5 min and a final extension step at 72°C for 7 min were also performed.

Each PCR product of EHMfas1 was directly sequenced with an ABI PRISM BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) on an ABI PRISM 3100-Avant Genetic Analyzer (Applied Biosystems), according to the manufacturer's directions. Each DNA sequence was analyzed several times using independent DNA preparations.

DNA sequences of chitinase and SREHP were aligned using GENETYX ver. 7.0.11 (Genetyx). The GenBank accession numbers of the sequences used for comparison with the chitinase and SREHP genes are shown in Figs. 1 and 2.

### Phylogenetic analysis

Analysis and multiple alignment of DNA sequences of 16S-like SSUrDNA were performed with ClustalX (Thompson et al. 1997), and the phylogenetic tree was constructed using the neighbor-joining method (Saitou and Nei 1987). The GenBank accession numbers and length of the sequences used in the phylogenetic analysis were as follows: X56991 (*E. histolytica*: 1,911 bp), X64142 (*E. histolytica* strain HM-1:IMSS: 1,910 bp), X61116 (*E. histolytica* strain SD4: 1,907 bp), Z49256 (*E. dispar*: 1,913 bp), AF149906 (*E. moshkovskii*: 1,911 bp), AF149910 (*E. terrapinae*: 1,910 bp), AF149909 (*E. insolita*: 1,909 bp), AF149907 (*E. hartmanni*: 1,927 bp), D28490 (*E. gingivalis*: 1,910 bp), AF149905 (*E. invadens*: 1,932 bp), AF149908 (*E. ranarum*: 1,901 bp), AF149912 (*E. chattoni*: 1,830 bp), AF149913 (*E. polecki*: 1,825 bp), AF149914 (*E. coli*: 2,068 bp), DQ286371 (*E. equi*: 1,886 bp), DQ286372 (*E. suis*: 1,970), DQ286373 (*E. ecuadoriensis*: 1,908 bp) and AJ566411 (*E. struthionis*: 1,830 bp). Comparisons were made over 1,911 bp of EHMfas1. However, as the submitted *E. suis* sequence lacked some homologous base pairs at the 5' and 3' termini, the GenBank sequences were aligned after deleting some terminal base pairs to allow a direct alignment.

### Nucleotide sequence accession numbers

The nucleotide sequence data reported here have been submitted to the GenBank/European Molecular Biology Laboratory/DNA Data Bank of Japan database under accession numbers AB282755 (chitinase), AB197935 (SREHP), and AB197936 (16S-like SSUrRNA).

## Results

### Polymorphism in the chitinase and SREHP loci of EHMfas1

The chitinase and SREHP genes of EHMfas1 were sequenced and compared with human isolates, which were

genotyped and classified by the combination pattern of repeating units in the polymorphic region (Haghighi et al. 2002, 2003; Figs. 1 and 2).

The 5'-conserved region, the repeat-containing polymorphic region, and the 3'-conserved region of the EHMfas1 chitinase gene were compared with those of human isolates. A number was tentatively assigned to each unit in the nucleotide and deduced amino-acid sequences (Fig. 1). In human isolates, the polymorphic region is constructed from combinations of one type of non-repeating unit, three types of repeating units, and two conserved regions (Haghighi et al. 2002). The nucleotide sequence of EHMfas1 consists of two types of non-repeating units (CN1 and CN7), three types of repeating units (CN2, CN3, and CN5), and the 5'- and 3'-conserved regions (CN5'C and CN3'C1; Fig. 1a). The CN1 and CN2 units were common to EHMfas1 and the human isolates, but CN3, CN5, CN7, and CN3'C2 were EHMfas1-specific mutated units; these units corresponded to the CN2, CN4,

CN6, and CN3'C1 units, respectively, in human isolates, with a single-nucleotide substitution in each unit (Fig. 1a). In the deduced amino-acid sequence, only the CA3'C2 had a single amino-acid substitution compared to the corresponding human units (Fig. 1b).

The 5'-conserved region, the repeat-containing polymorphic region, and the 3'-conserved region of the EHMfas1 SREHP gene were also compared with those of human isolates. As for chitinase, a number was tentatively assigned to each unit in the nucleotide and deduced amino-acid sequences (Fig. 2). In human isolates, the polymorphic region of the SREHP gene contains combinations of four types of non-repeating units, five types of repeating units, and two conserved regions; some of the human isolates also have strain-specific mutated units (Haghighi et al. 2002, 2003). The nucleotide sequence of EHMfas1 consists of six types of non-repeating units (SN1, SN2, SN5, SN16, SN17, and SN20), four types of repeating units (SN3, SN9, SN10, and SN11), and the 5'- and 3'-conserved regions (SN5'C and

**Fig. 1** Schematic representation of polymorphism in the repeat-containing region of the chitinase gene among EHMfas1 and human isolates. Nucleotide sequences pattern (a) and deduced amino-acid sequences pattern (b) were shown. Each nucleotide sequence of unit was tentatively given a number. Nucleotide and deduced amino-acid sequences of these units are also shown. Enclosed units with bold line were EHMfas1-specific units. EHMfas1-specific mutations in nucleotide and deduced amino-acid sequences are underlined. Superscript letter a EHMfas1-specific unit sequences

