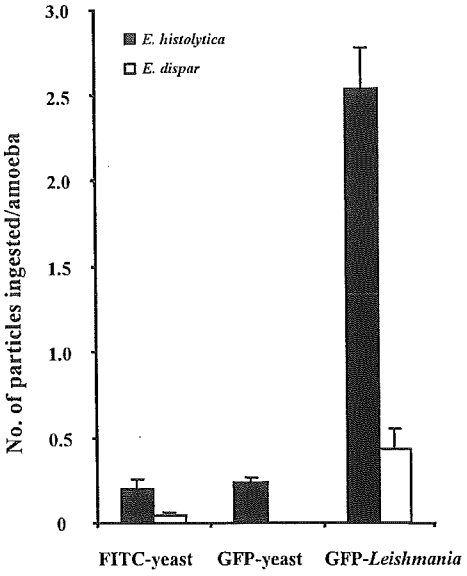

Fig. 3. Phagocytosis of FITC-yeast, GFP-yeast, or GFP-*L. amazonensis* by *E. histolytica* (filled bars) and *E. dispar* (open bars), respectively. Parasites were incubated with opsonised FITC-conjugated yeasts, live GFP expressing-yeasts, or GFP-expressing *Leishmania* promastigotes at a 1:20 ratio at 33°C for 20 min, captured images, counting the ingested cells per amoeba. The value is the average of three independent experiments. Error bars represent the S.D. of the measurement of 250–300 particles.

cytes and beads not shown) in both *E. histolytica* and *E. dispar*. However, the efficiency was ~6-fold higher in *E. histolytica* than in *E. dispar*. *E. dispar* failed to ingest the live GFP-yeasts, even after 3 h of incubation (data not shown).

Distinct Kinetics of Phagosome Acidification in *E. histolytica* and *E. dispar*

To examine the kinetics of phagosome acidification, we used opsonized yeasts covalently labeled with pH-sensitive FITC, and measured the phagosome pH with ratiometry on high-resolution video microscopy. Trophozoites attached on the glass surface were incubated with and allowed to ingest FITC-yeasts. We captured images of trophozoites that ingested single yeast at 30 s intervals for 3 h to monitor the phagosome pH. Upon internalization of yeast particles, phagosomes of F4 *E. histolytica* were acidified very rapidly (Fig. 4A). Within 2 min, the pH of phagosomes decreased from that of the extracellular medium (6.70 ± 0.20) to 4.58 ± 0.36 in *E. histolytica*. The phagosome remained acidified (4.54 ± 0.16) for at least 12 h (only data up to 30 min are shown). In *E. dispar*, phagosomes were less acidified than in *E. histolytica*. Upon ingestion, the pH dropped to 5.83 ± 0.38 after 2 min and remained at 5.60 ± 0.19 up to 12 h in *E. dispar* (only data up to 30 min are shown). The phagosome acidification occurred at a higher rate in *E. histolytica* than in *E. dispar*; the initial acidification occurred at the rate of a decrease of 1.06 and 0.44 pH/min for the first 2 min in *E. histolytica* and *E. dispar*, respectively.

Phagosome Acidification of *E. histolytica* and *E. dispar* is Mediated by V-ATPases

To see whether V-ATPase is involved in phagosome acidification in *Entamoeba*, we examined the effects of concanamycin A, a potent and specific proton pump inhibitor [Rathman et al., 1996; Hackam et al., 1997; Arora et al., 2000]. One hour pretreatment of trophozoites with 10 μM concanamycin A almost completely abolished phagosomal acidification both in *E. histolytica* and *E. dispar* (Fig. 4B). The pH of phagosomes remained at 6.18 ± 0.18 or 6.15 ± 0.23 in *E. histolytica* or *E. dispar*, respectively. These results are consistent with the premise that V-ATPases play an essential role for the initial acidification of phagosomes and the maintenance of acidification in both species.

Inhibition of Microtubules, Actin, or Myosin Affects the Kinetics of Acidification in *E. histolytica*

To investigate whether microtubules were required for phagosome acidification, we treated trophozoites with 20–200 μM nocodazole [Scheel et al., 1990; Bayer et al., 1998], or 100–200 μM oryzalin [Makioka et al., 2000], which are known to depolymerize microtubules, for 1 h and then allowed trophozoites to ingest FITC-yeasts. The acidification of phagosomes was slightly hindered by nocodazole (Figs. 4B and 5A) or oryzalin F5 (Fig. 5B) in both *E. histolytica* and *E. dispar*. The pH reached between 5.17 and 5.21 in the *E. histotytica* trophozoites treated with the highest concentrations of the drugs (Fig. 5A and 5B), while it reached 5.78 ± 0.18 in *E. dispar* treated with 20 μM nocodazole (Fig. 4B) (the results of treatment of *E. dispar* with oryzalin was not shown).

We also tested whether actin or myosin is also involved in phagosome acidification in *E. histolytica*. Treatment of trophozoites with 0.25–0.5 μM of latrunculin A, an actin inhibitor [Makioka et al., 2001], resulted in the immobilization of trophozoites and detachment from the slide glass. The ingestion of the FITC-yeast particle was also significantly reduced (data not shown). However, phagosome acidification was only slightly inhibited (pH 4.97 ± 0.08) (Fig. 5C). Treatment of trophozoites with 20–40 mM BDM, a myosin inhibitor, gave the most notable reduction of acidification (Fig. 5D, pH 5.33 ± 0.11). The initial phase of the rapid decrease in pH, in particular, was significantly perturbed. The initial acidification occurred at the rate of 0.36 pH

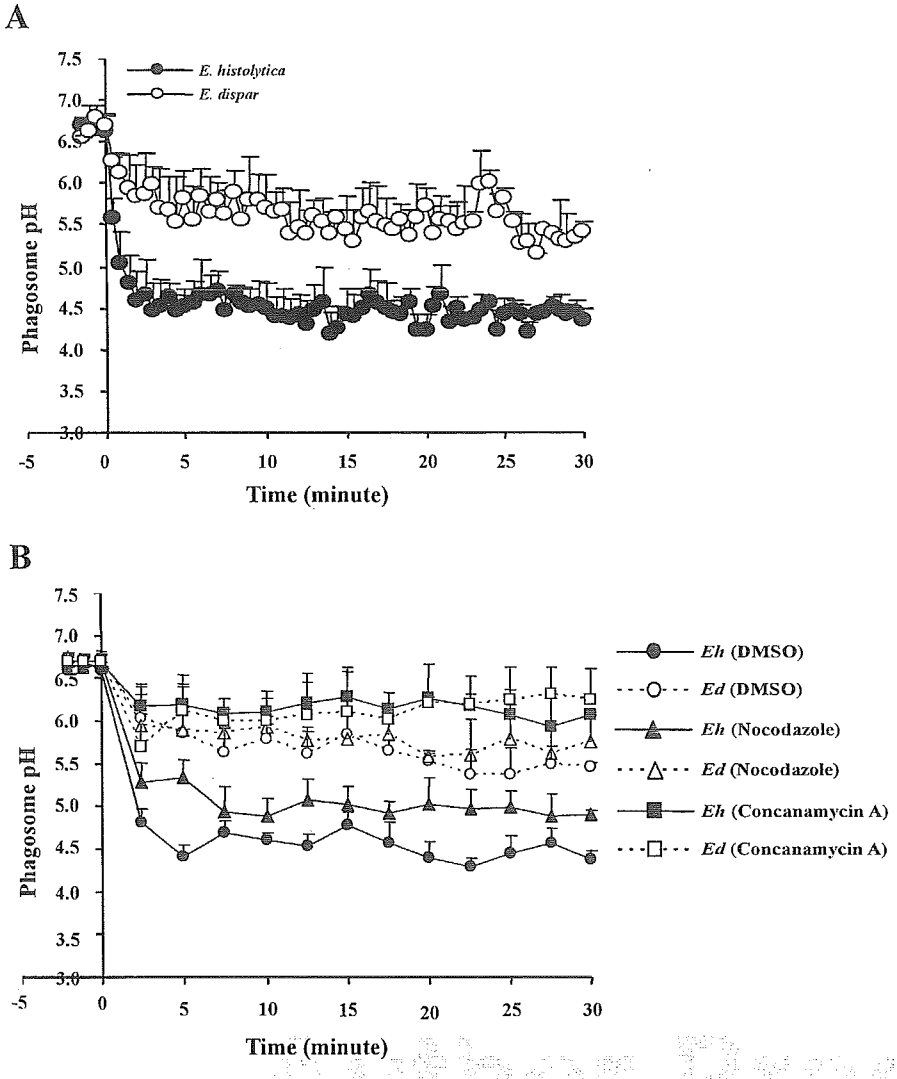

Fig. 4. Kinetics of acidification during phagosome maturation. **A**: The changes of phagosome pH in *E. histolytica* (closed circles) and *E. dispar* (open circles). Trophozoites were mixed with FITC-yeasts at a 1:10 ratio on a glass bottom culture dish, enclosed with a cover slip, and allowed to ingest yeast particles at 33°C. Trophozoites containing a single particle were selected and phagosome pH was measured at 30 s intervals for 3 h with time-lapse video microscopy on a Leica AS MDW, as described in the MATERIALS AND METHODS. Only data for the first 30 min are shown. Error bars represent the S.D. of 10 independent phagosomes. **B**: Effects of concanamycin A and nocodazole on phagosome acidification in *E. histolytica* (filled symbols and unbroken lines) and *E. dispar* (open symbols and dotted lines). Trophozoites were pretreated with 10 μM concanamycin A (squares), 20 μM nocodazole (triangles), or DMSO only (circles) for 1 h, washed, and incubated, and the pH was determined as described above, except for the time intervals (2.5 min). Error bars represent the S.D. of eight independent phagosomes.

decrease per minute for the first 2.5 min in the trophozoites treated with 40 mM BDM, while it occurred at 0.56, 0.60, or 0.56 in the cells treated with a highest concentration of nocodazole, oryzalin, or latrunculin A, respectively.

Distinct Kinetics of Degradation of GFP-Yeasts or GFP-*L. amazonensis* Promastigotes in Phagosomes Between *E. histolytica* and *E. dispar*

We next examined the kinetics of disintegration of ingested particles followed by degradation of a marker GFP by measuring the fluorescence signal of GFP expressed by live yeasts or promastigotes of *L. amazonensis*. A notable difference in the kinetics of GFP degradation was observed in *E. histolytica* between GFP-yeasts and GFP-*Leishmania* (Fig. 6A). While the fluorescence of GFP-*Leishmania* diminished rapidly (94.5% ± 0.71% of the initial GFP fluorescence was lost in the first 30 min), that of GFP-yeasts was more stable (only 26% GFP fluorescence signal disappeared in 30 min). Since GFP-expressing *Leishmania* is the best prey for the amoeba trophozoites because of its high internalization rate and stable high fluorescence compared with GFP-expressing bacteria or yeasts, we used GFP-expressing *Leishmania* promastigotes as a model to evaluate degradation in phagosomes. Degradation of GFP-*Leishmania* in *E. dispar* was slower and less efficient than in *E. histolytica*. *E. dispar* degraded only 30% of the initial fluorescent signal of ingested GFP-*Leishmania* in 30 min (Fig. 6A). As *E. dispar* failed to ingest live GFP-yeasts, the kinetics of GFP-yeast degradation was not determined in *E. dispar*.

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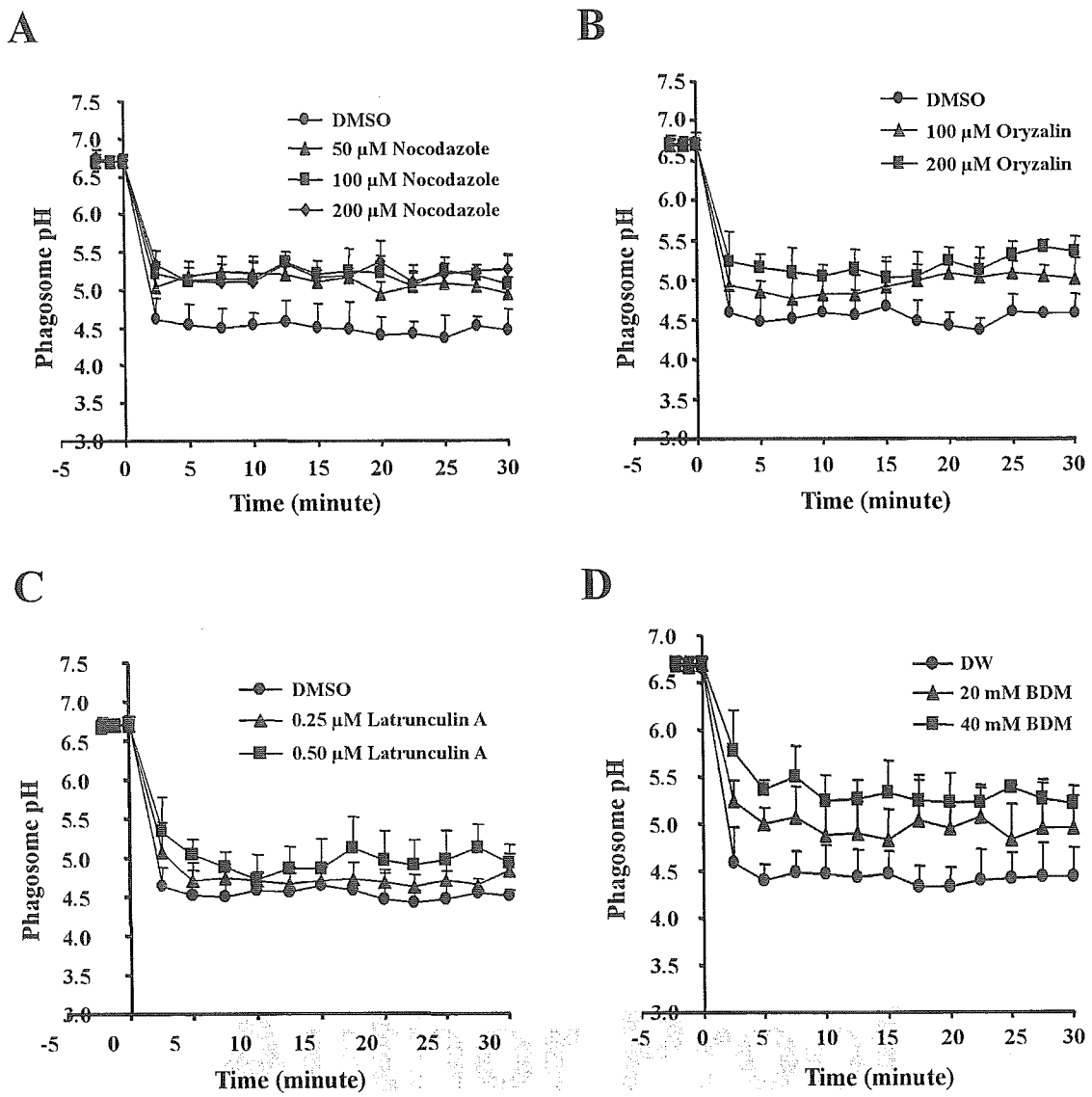
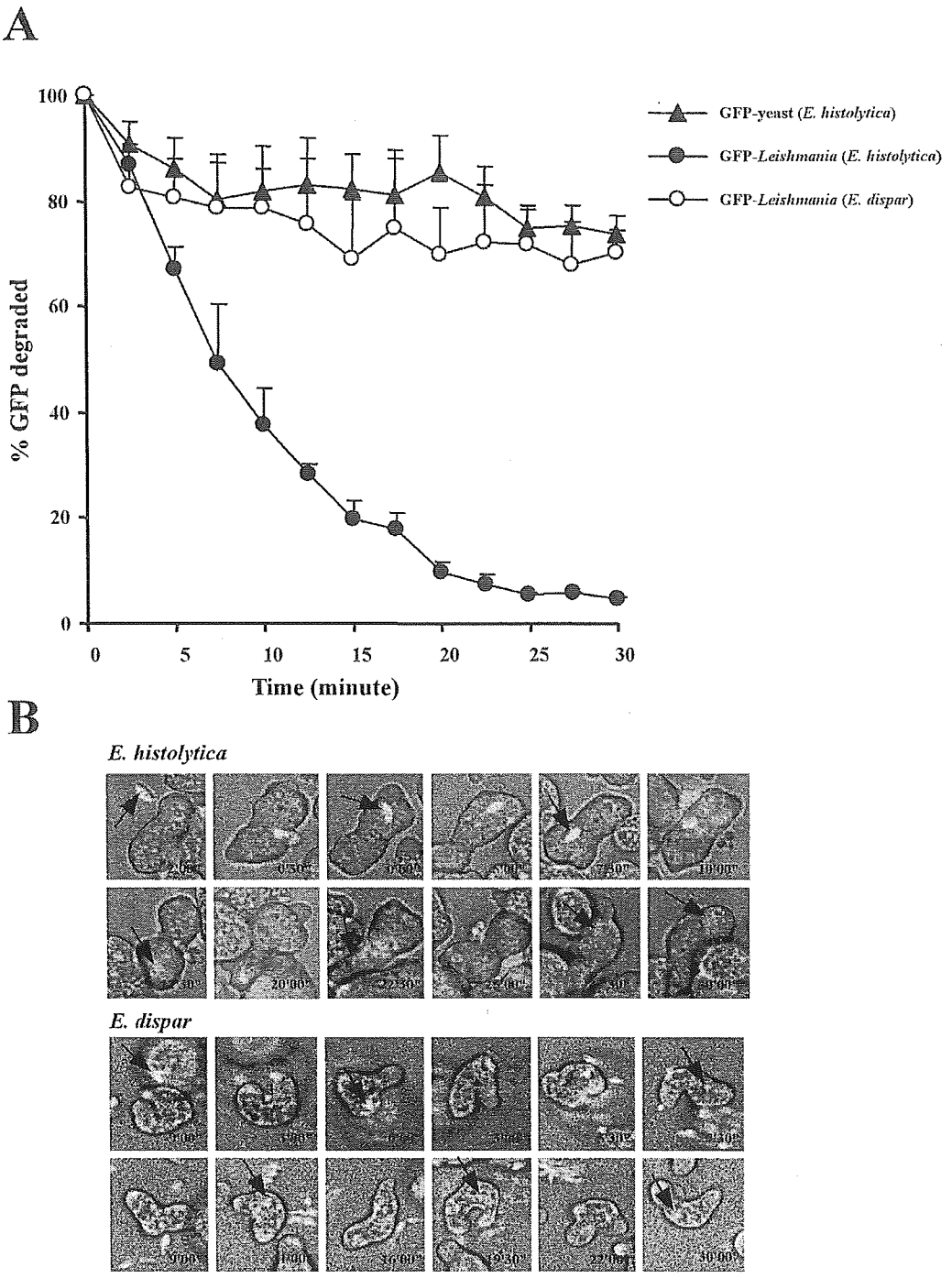


Fig. 5. Effects of different concentrations of nocodazole, oryzalin, latrunculin A and BDM on phagosome acidification in *E. histolytica*. Trophozoites were pretreated with 0, 50, 100, or 200 μM of nocodazole (circles, triangles, squares, or diamonds, respectively) (**A**); 0, 100, or 200 μM of oryzalin (circles, triangles, or squares, respectively) (**B**); 0, 0.25 or 0.50 μM latrunculin A (*circles, triangles, or squares, respectively*) (**C**); and 0, 20, or 40 mM BDM (circles, triangles, or squares, respectively) (**D**). DW represents distilled water. Error bars represent S.D. of 6–10 phagosomes.

Acidification of Phagosomes is Required for the Efficient Degradation of GFP-*L. amazonensis*

We determined whether acidification is required for GFP degradation within phagosomes. *E. histolytica* and *E. dispar* trophozoites were pretreated with 10 μM concanamycin A for 1 h, and were then allowed to ingest live GFP-*L. amazonensis*. In both *E. histolytica* and *E. dispar*, the degradation of GFP-*Leishmania* was significantly inhibited; after concanamycin A treatment, only 44.0 ± 1.4 or 1.5% ± 2.1% of the initial GFP signal was lost in 30 min in *E. histolytica* and *E. dispar*, respectively (53% or 95% relative reduction, respectively). Thus, the acidification of phagosomes is required for efficient degradation in phagosomes in both species, as shown for mammalian cells [Arora et al., 2000]. Although acidification was almost completely abolished by concanamycin A treat-



COLOR

Fig. 6. Degradation of GFP-expressing yeasts and *Leishmania* promastigotes in *E. histolytica* (filled symbols) and *E. dispar* (opened symbols). **A:** Trophozoites of *E. histolytica* or *E. dispar* were incubated with live GFP-yeasts or GFP-*Leishmania* promastigotes at a 1:10 ratio and images were captured at 30 s interval to measure the GFP fluorescence intensity. Only data of 2.5 min intervals were shown. Error bars represent the S.D. of 7–8 independent phagosomes. **B:** Sequential images of representative experiments showing the degradation of GFP-*L. amazonensis* promastigotes in an *E. histolytica* (top panels) or *E. dispar* (bottom panels) trophozoite. Arrows indicate the GFP-*Leishmania* promastigotes. Time (min, s) is shown at the bottom.

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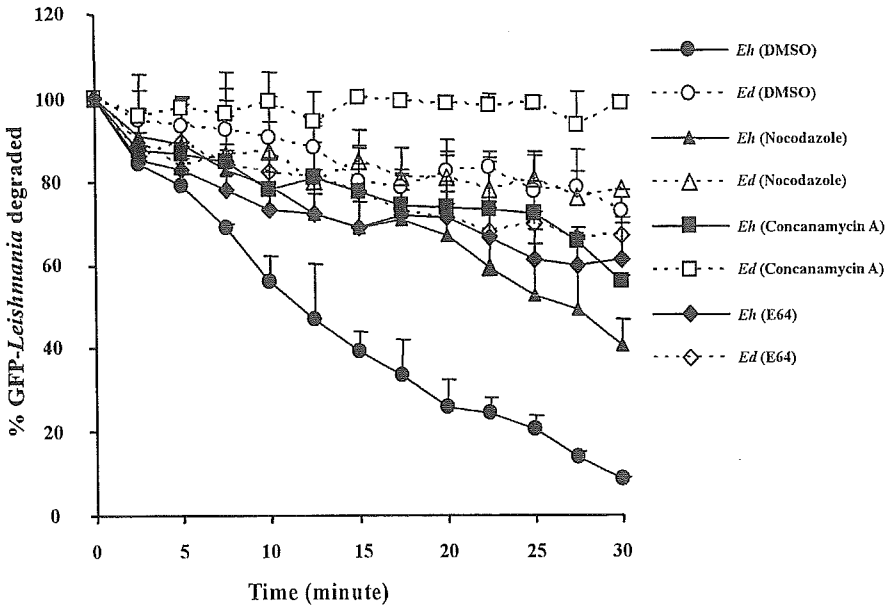

Fig. 7. Effects of inhibitors on the degradation of GFP-expressing *Leishmania* promastigotes in *E. histolytica* and *E. dispar*. Trophozoites were pretreated with concanamycin A, E64, or nocodazole for 1 h, washed and mixed with live GFP-*Leishmania* promastigotes. Degradation was monitored as in Fig. 6A. The fluorescent intensities of 10 independent phagosomes were measured.

ment in both species, the inhibition of GFP-*Leishmania* was more pronounced in *E. dispar* than in *E. histolytica*. This may suggest the unique presence of an alternative degradation machinery that does not require acidification for its activation in *E. histolytica*.

Inhibition of CP or Microtubules Hampers GFP Degradation in *E. histolytica* but Not in *E. dispar*

Inhibition of CPs by an irreversible, potent, and highly selective CP inhibitor E64 [Katunuma and Kominami, 1995; Sreedharan et al., 1996] blocked the degradation of GFP-Leishmania only in *E. histolytica* but not in *E. dispar* (Fig. 7). In 30 min, only 39.0% ± 8.7% of GFP-*Leishmania* was degraded (59% reduction) in *E. histolytica*, while E64 showed almost no effect on the degradation in *E. dispar*. These data suggest the presence of not-yet-identified digestive enzymes other than CP for degradation in phagosomes of *E. dispar*.

Disruption of microtubules by nocodazole significantly inhibited the GFP degradation in *E. histolytica* (at 30 min only 59% GFP was degraded; 38% reduction), which indicates the importance of microtubules in degradation in phagosomes of *E. histolytica* (Fig. 7). In contrast, in *E. dispar*, nocodazole showed no significant effect on degradation.

DISCUSSION

Morphological Differences in Phagosomes Between Virulent and Avirulent *Entamoeba* Species

Electron micrographs of the trophozoites of axenized *E. histolytica* and *E. dispar* strain cultivated with *P. aeruginosa* revealed significant differences in both the number of bacteria in a single phagosome and the average diameter of phagosomes between these two species (Figs. 1 and 2). Previously, it was shown that *E. dispar* trophozoites contain only a single bacterium in a single phagosome and a fraction of bacteria were not enclosed by membrane structures in the cytoplasm [Pimenta et al., 2002]. Our results strongly argue against these observations: all ingested bacteria were enclosed in the phagosomal membrane and phagosomes containing multiple bacteria were found in *E. dispar*. Phagosomes of *E. histolytica* are significantly larger in size and contained a higher average number of bacteria than those in *E. dispar*. These morphological differences are likely associated with differences in the kinetics of phagosome acidification and the efficiency of degradation of ingested substances in phagosomes demonstrated between these two species (see below).

Differences in the Kinetics of Phagosome Acidification Between Virulent and Avirulent *Entamoeba* Species and Between *Entamoeba* and Other Organisms

We have demonstrated the kinetics of acidification of individual phagosomes (not as a whole cell) in *Entamoeba* using highly sensitive and high-resolution video microscopy and the pH-sensitive FITC-labeled yeasts. Differences in the efficiency of phagosome acidification between the two *Entamoeba* species indicate the significant differences in nature of proton pumping across the phagosome membrane and membrane trafficking, leading to phagosome maturation. The abrupt pH

decrease upon ingestion observed in both species suggests that direct recruitment of V-ATPase to a newly formed phagosome or, more likely, swift recruitment and fusion of lysosomes in the initial phase of phagosome biogenesis, as previously shown in macrophages and neutrophils [Styrt and Klempner, 1982; Lukacs et al., 1990]. Acidification of endosomes was also previously studied in *E. histolytica* [Meza and Clarke, 2004] and *Dictyostelium discoideum* [Clarke et al., 2002a]. A very rapid acidification of phagosomes we demonstrated in the present work is in very good contrast to the rather slow acidification of endosomes in *E. histolytica* [Meza and Clarke, 2004]. In contrast, the persistence of the acidified environment is shared by both phagosomes and endosomes, suggesting the presence of a common mechanism for the sustained acidification of these compartments.

The pH values of the phagosome and its homologous compartment (digestive vacuole) reported for mouse macrophages, hemocytes of *Mytilus edulis*, *amoeba proteus*, and *D. discoideum* vary in a range of 4.5–5.3 [Geisow et al., 1981; Kroschinski and Renwrantz, 1988; Rupper et al., 2001]. In these organisms, the acidification of phagosomes occurred within 7–15 min after ingestion [Jensen and Bainton, 1973; Geisow et al., 1981; Bassoe and Bjerknes, 1985; Rupper et al., 2001]. The acidification of phagosomes in *Entamoeba* occurred more rapidly (within 2 min) than these organisms (Fig. 4A).

The persistence of acidity in phagosomes for >12 h in *Entamoeba* was surprising, as the maintenance of an acidic pH is energetically expensive due to the continuous expenditure of ATP. It has been shown in a few organisms, including *D. discoideum*, that following the rapid acidification of phagosomes upon the internalization of external particles, neutralization of phagosomes occurs after 30–60 min [Rupper et al., 2001]. It was also shown that the digestive vacuole of *Paramecium caudatum* began to neutralize within 8 min after internalization [Fok et al., 1982]. Thus, *Entamoeba* may represent a unique organism that sustains phagosome acidity for an unusually long period, which may be associated with its high capacity for phagocytosis and degradation. The sustained acidity of the *Entamoeba* phagosomes indicates the continuous presence of V-ATPase on the phagosome membrane or, alternatively, the existence of unidentified molecules for the maintenance of a pH gradient across the phagosomal membrane in *Entamoeba*. We should also mention that ~30%–35% of the ingested yeasts were expelled by *E. histolytica* trophozoites without complete degradation between 90 and 120 min after internalization [Mitra and Nozaki, unpublished]. We reproducibly observed that a small (0.5–0.7) pH increase occurred <5 min prior to expulsion (data not shown). Taken together, the persistent acidification of phagosomes may occur only in cases where the degradation in phagosomes is not completed, such as in inert latex particles or fixed bioparticles.

V-ATPase is Essential for the Phagosome Acidification in both *Entamoeba* Species

Phagosome acidification was completely blocked with the V-ATPase inhibitor in both *Entamoeba* species, similar to mammalian cells, as previously shown [Arora et al., 2000]. These data, together with our demonstration of most of the homologs of V-ATPase subunits (i.e., A–H subunits of V_1 subcomplex and a–c subunits of V_0 subcomplex, data not shown) in the genome databases of the both species (http://www.tigr.org/tdb/e2k1/eha1/; http://www.sanger.ac.uk/Projects/E_dispar/), indicate that V-ATPase is primarily involved in the initial acidification and the maintenance of acidic phagosomes in both *Entamoeba* species, as shown for *D. discoideum* [Rezabek et al., 1997]. Some of these V-ATPase subunits were also previously identified from *E. histolytica* [Descoteaux et al., 1994]. In addition, our proteomic analysis of phagosomes purified with sucrose step gradient centrifugation using carboxylated latex beads also revealed three subunits of the V-ATPase V_0 subcomplex (a, b, and c) and proteolipid [Okada et al., 2005]. The fact that the inhibition of V-ATPase severely retarded the degradation of GFP expressed by the ingested *Leishmania* promastigotes indicates that acidification is essential for degradation within phagosomes.

It was shown that the inhibition of V-ATPase by bafilomycin caused a reduction in phagocytosis [Ghosh and Samuelson, 1997]. It was also shown that V-ATPase (Vph1p) is involved in the virulence of the AIDS-related opportunistic fungal pathogen, *Cryptococcus neoformans* [Erickson et al., 2001]. Disruption of *VPH1* resulted in defects of four virulence factors, i.e., capsule, laccase, and urease, and also growth [Erickson et al., 2001]. Thus, V-ATPase seems to play a variety of roles in the virulent mechanisms of pathogens.

Involvement of Microtubules, Actin, and Myosin in the Phagosome Maturation

It is well established in general that the actin cytoskeleton is essential for phagosome formation. Recent data suggest that phagosome-bound actin is lost soon after phagosome formation [Greenberg et al., 1991; Rupper et al., 2001], indicating the role of the actin-rich cytoskeleton in the early stages of phagosome maturation. In *E. histolytica*, it has been demonstrated that F-actin is present around phagosomes and colocalized with myosin IB [Voigt et al., 1999]. Voigt et al. also showed that myosin IB was also present in association with phagosomes at later stages of phagocytosis, suggesting its constitutive role in phagosome maturation [Voigt et al., 1999]. Our study, using specific inhibitors against microtubules, actin and myosin, indicates that myosin plays a significant role in phagosome acidifica-

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tion of this parasite, while microtubules and actin, which are inhibitable by conventional inhibitors, are not directly involved in the process. Our study also suggests that additional unidentified mechanisms exist for phagosome acidification in this organism.

Microtubules have been believed to play an essential role in phagosome maturation through cytoskeletal reorganization and facilitating fusion between the phagosomes and endosomes/lysosomes in various organisms, e.g., [Desjardins et al., 1994; Blocker et al., 1996; Clarke et al., 2002a]. However, in *E. histolytica*, the absence of obvious microtubule-like structures in the cytoplasm, as demonstrated by EM, suggests that the cytoplasmic microtubules of this parasite may be particularly labile to fixation techniques known to preserve microtubules in other eukaryotic cells, or alternatively, microtubules are present only in the nuclei [Vayssie et al., 2004]. It was previously demonstrated that the transport of V-ATPase to early endosomes in *Dictyostelium* occurs by the rapid fusion of endosomes mediated by microtubules [Clarke et al., 2002a, b]. In macrophages, the depolymerization of microtubules severely affected degradation of opsonized *Staphylococcus aureus* [Damiani and Colombo, 2003]. As the two *Entamoeba* species revealed similar morphological features on the cytoskeleton (this study), the differential effects of the microtubule inhibitor on degradation in phagosomes between the two species were unexpected. Although the microtubule inhibitors only slightly reduced the phagosome acidification, nocodazole notably reduced degradation in phagosomes of *E. histolytica*, while almost no inhibition of phagosome acidification or degradation was found in *E. dispar*. In the mouse, one of the V-ATPases, which contains the *a3* isoform among the four isoforms (*a1–a4*) of V_0 *a* subunit [Toyomura et al., 2000], has been shown to localize to late endosome/lysosomes, and be associated with microtubules and lysosomal marker protein, Lamp2 (Toyomura et al., 2003), as shown by the depolymerization of microtubules resulting in the dispersion of *a3*-associated V-ATPase and Lamp2 [Toyomura et al., 2003]. So, a disparity in the effects on microtubules and V-ATPase in *E. dispar* may suggest a lack of interaction between microtubules and V-ATPase in *E. dispar*. Alternatively, *E. dispar* was relatively resistant to nocodazole, compared to *E. histolytica*. It was previously shown that the sensitivity to nocodazole varied among cell types [Gruenberg and Howell, 1989; van Deuer et al., 1995]. It has also been shown that the disassembly of microtubules by nocodazole depends on the temperature and duration of treatment [Cirillo et al., 1999].

Involvement of CP in Degradation in Phagosomes

It is conceivable to speculate that acidification for efficient degradation within phagosomes is essential for the activation of hydrolytic enzymes. *Entamoeba* kills or digests bacteria and host cells (in case of *E. histolytica*) within their phagolysosomes via oxygen-independent mechanisms, using a variety of digestive proteins, including CP, lysozymes, and amoebapores [Bruchhaus et al., 1996; Leippe, 1997]. It was shown that the digestion of internalized collagen in fibroblasts largely depends on the activity of CP, e.g., cathepsin B [Everts et al., 1996]. Acidification triggers amoebapore activation in *E. histolytica* [Bruhn et al. 2003]. Our proteomic analysis of phagosome proteins in *E. histolytica* revealed a variety of hydrolytic enzymes and degradative proteins including, CP1, 2, 4 and 5, phospholipases, dipeptidylaminopeptidase, β-hexosaminidase, and lysozymes [Okada et al., 2005]. It is conceivable that continuous acidification is required for the activation of these various hydrolytic enzymes, which are recruited at different times (Okada et al, unpublished) in the course of phagosome maturation.

Degradation in the *E. histolytica* phagosomes occurs somewhat faster (~3–4 fold) compared with the *E. dispar* phagosomes, which is consistent with the previous finding that the CP activity in *E. histolytica* is 10–1000-fold higher than in *E. dispar* (Bruchhaus et al., 2003). This also indicates, together with our finding that the CP inhibitor did not inhibit degradation in phagosomes of *E. dispar* that CP may not be the major protease for digestion in phagosomes in this species. It is also possible that the active site assembly of CPs has different requirements depending on the type of CPs and that E64 selectively binds to substrates with a particular conformation of the active site. Therefore, it is conceivable that some of CPs from *E. dispar* might be relatively resistant to E64. Altogether, differences in the efficiency of internalization, degradation in phagosomes, and enzymes involved in the degradation between the two species are likely associated with the efficiency of removal and degradation of human cells on intestinal mucosa, which partially determines the outcome of infection.

Efficient *Leishmania* Degradation in *Entamoeba* Phagosomes

We have shown that *Entamoeba* trophozoites are able to internalize and degrade *Leishmania* promastigotes more efficiently than yeasts (Fig. 5A) or erythrocytes (data not shown). It is known that *Leishmania* is able to protect itself from phagolysosome degradation in macrophages by inhibiting phagosome-endosome fusion [Desjardins and Descoteaux, 1997], hydrolytic enzymes [Sacks et al., 2000], cell signaling pathways [Cunningham, 2002], nitric oxide production [Holm et al., 2001] and cytokine production [Piedrafita et al., 1999] with the lipophosphoglycan molecule. Thus, the inability of *Leishmania* promastigote's survival in *Entamoeba* suggests that the target proteins of *Leishmania* lipophospho-

glycan or downstream effectors that *Leishmania* utilizes for the impediment of phagosome maturation are absent in *Entamoeba*. Conversely, *Entamoeba* possesses a receptor to interact with the *Leishmania* cell surface, leading to the activation of a downstream signaling pathway necessary for further phagosome maturation. Since the *Entamoeba* parasite does not presently encounter *Leishmania* in its life cycle, the intestinal microorganisms that *Entamoeba* normally ingests in mammalian intestines likely possess ligands with structures shared by *Leishmania*.

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