Resistance of *Entamoeba histolytica* to the cysteine proteinase inhibitor E64 is associated with secretion of pro-enzymes and reduced pathogenicity

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Running title: E64-resistance of *E. histolytica*

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SUMMARY

Cysteine proteinases (CPs) have been considered as suitable targets for the development of antiparasitic drugs. To assess the importance of CPs for the growth and pathogenicity of the protozoan parasite Entamoeba histolytica we have cultured amoebae in the presence of various cysteine proteinase inhibitors (CPIs). It was found that broad range CPIs, which are membrane permeable and rapidly enter the cell, are highly toxic at micromolar concentrations and all attempts to generate E. histolytica mutants resistant to these CPIs were unsuccessful. In contrast, the broad-range CPI E64, which does not permeate membranes as well, was deleterious at much higher concentrations and amoebae rapidly developed resistance to this inhibitor. Compared to sensitive wild-type cells, E64-resistant E. histolytica were substantially reduced in the expression of various CP genes and were able to secrete unprocessed enzyme into the culture medium. Moreover, E64-resistance was associated with a significant reduction in virulence, as these cells were greatly impaired in the ability to generate liver abscesses in experimentally infected gerbils.
INTRODUCTION

Cysteine proteinases (CPs) are important virulence factors of various infectious agents and the main proteolytic enzymes in many protozoan parasites (1,2). An attractive aspect of CPs is their widespread importance in the metabolism of all organisms studied so far. Accordingly, CPs have been considered as promising drug targets for the development of antiparasitic chemotherapy (3-5). During recent years, numerous studies have been performed to evaluate the antiparasitic effect of CP inhibitors (CPIs), which indicated that CPIs show indeed potent in vitro and in vivo growth inhibitory activity against various protozoans such as Plasmodium, Trypanosoma or Leishmania species (6-19).

The intestinal protozoan parasite Entamoeba histolytica, the causative agent of human amoebiasis is characterized by its high capacity to destroy host tissues, leading to potentially life-threatening diseases such as ulcerative colitis or liver abscess. E. histolytica is known to produce considerable amounts of CPs and convincing evidence exists that CPs are instrumental for E. histolytica-induced pathology. At least 20 cp-gene exist in the E. histolytica genome. Interestingly, the majority of the various CPs are not expressed in E. histolytica trophozoites during in vitro cultivation. Northern blot analyses indicated that ehcp1, ehcp2 and ehcp5 showed the strongest expression of all proteinase genes leading to approximately 90% of total cp-transcripts (20). Treatment of amoebae with sublethal doses of the CPI E64 or the addition of laminin, which blocks the substrate-binding pocket of CPs, greatly reduced the ability to produce liver abscesses in laboratory animals (21,22). Likewise, liver abscess formation was totally blocked, and significantly less gut inflammation and damage to the intestinal permeability barrier were observed when
animals were infected with amoebae, which were genetically engineered to produce low levels of CP activity (23-25).

For treatment of amoebic diseases, such as colitis or liver abscess, metronidazole is the drug of choice, which has been used in clinical practice for more than 25 years. At present, treatment of E. histolytica with metronidazole is still very effective but recently in vitro-induced metronidazole resistance of E. histolytica trophozoites has been reported (26,27). In addition to drugs that are directed against amoebae that have invaded into the tissue, there is need for more effective luminal anti-amoebic agents to eliminate the parasite from the intestine, which is important to interrupt transmission and to prevent E. histolytica carriers from progression into disease (28JCM,29). A recent study indicated that the efficacy of the two drugs presently available for the treatment of E. histolytica carriers was only 87% and 57%, respectively (30).

To evaluate whether E. histolytica CPs constitute suitable targets for the development of anti-amoebic drugs we have cultured the parasite in the presence of various CPIs and have characterized cells that were resistant to E64.
EXPERIMENTAL PROCEDURES

**Chemicals**—For *E. histolytica* growth inhibition as well as inhibition of cysteine proteinase activity the CPIs E64 (L-trans-epoxysuccinyl-leu-4-guanidinobutylamide, Sigma), E64d (L-trans-epoxysuccinyl (OEt)-leu-3-methylbutylamide-ethyl ester, Sigma), Z-Phe-Ala-DMK (Z-Phe-Ala-diazomethylketone, Bachem), Z-Phe-Phe-DMK (Z-Phe-Phe-diazomethylketone, Bachem) and p-HMB (para-hydroxymercuribenzoat, Sigma) were used.

**Cultivation of cells**—Trophozoites of the *E. histolytica* isolate HM-1:IMSS were cultured axenically in TYI-S-33 medium in plastic culture flasks (31). The culture medium was changed every other day. If the amoebae were long term cultivated in the presence of E64, the inhibitor was re-supplied each time to the new culture medium.

For cultivation of the amoebae in the presence of different CPIs and for generation of E64-resistant amoebae, 1000 or 2000 amoebae/well were cultivated in the presence of the respective CPI under anaerobic conditions using Anaerocult A (Merck) in a 96 or 24 well plate, respectively. For detection of secreted proteins in the culture supernatant, the amoebae were cultivated for 3 h in the presence of phosphate buffered saline (6.7 mM Na$_2$HPO$_4$, 3.3 mM NaH$_2$PO$_4$, 140 mM NaCl, pH 7.2, PBS) instead of TYI-S-33 medium.

Trophozoites of cultures in the late logarithmic phase were harvested after being chilled on ice for 10 min and sedimented at 430 g at 4°C for 5 min. The resulting pellet was washed twice either in PBS or in TYI-S-33 medium without serum. For preparation of soluble extracts of amoebae, cells were lysed by four freeze-thaw
cycles in CO\textsubscript{2}/ethanol and sedimented by centrifugation at 15,000 g at 4°C for 15 min.

Chinese hamster ovary cells were grown in RPMI in the presence of 10% fetal calf serum, penicillin (100 U/ml) and streptomycin sulphate (199 µg/ml). Cells were released by trypsinization (0.05% trypsin/0.02% sodium EDTA) and sedimented at 250 g at room temperature for 3 min.

**Quantification of viable amoebae**—1000 amoebae/well were cultivated in a 96 well plate in the presence of different CPIs. After different time points of cultivation the amoebae were washed two times with prewarmed PBS (37°C) to remove dead cells. Subsequently, 10 µl H\textsubscript{2}O was added to each well and the plate was frozen at –70°C for cell lysis. After thawing of the plates at RT, the amount of protein/well was determined using the BCA (bichinonic acid reagent, Pierce) assay with BSA (Pierce No. 23210) as standard. The growth relative to the control (amoebae cultivated without CPI) was determined.

**Northern blot analysis**—Isolation of RNA from *E. histolytica* trophozoites was performed using TRIZOL reagent (Gibco BRL). For Northern blotting, agarose gels were loaded with 20 µg of total RNA. After transfer to a nylon membrane, the blots were sequentially hybridized with radiolabeled DNA probes obtained by restriction digest or PCR amplification of *E. histolytica* cysteine proteinases and the *E. histolytica* actin gene. Hybridizations were performed in 0.5 M Na\textsubscript{2}HPO\textsubscript{4}, 7% SDS and 1 mM EDTA (pH 7.2) at 55°C. Blots were washed in 40 mM Na\textsubscript{2}HPO\textsubscript{4} and 1% SDS (pH 7.2) at 55°C.
Protein analyses—12% SDS/PAGE was performed under reducing conditions. Western blot analyses were carried out by use of the semidry blotting technique with 10 mM 3-(cyclohexylamino)-1-propanonesulphonic acid (CAPS), pH 11.0/10% methanol as transfer buffer. For Western blot analyses, antibodies specific for EhCP1, EhCP2 and EhCP5 were used. Protein concentration was determined using bichinonic acid reagent (Pierce) and bovine serum albumin.

Enzymatic assays—Proteolytic activity was measured using the synthetic peptide Z-Arg-Arg-pNA (Bachem) as substrate. One unit of enzymatic activity is defined as the amount that catalyzes the reduction of 1 µmol/min of p-nitroaniline. For detection of proteinase activity by substrate gel electrophoresis, a 12% SDS polyacrylamide gel was copolymerized with 0.1% gelatin and subsequently treated as described.

Assay for determination of cytopathic activity—Interaction of trophozoites and CHO cells was determined by a modified method of Bracha and Mirelman (1984). CHO cells (1 x 10⁵) were grown to confluence in 24-well plates. After washing of the CHO cells with PBS, 1 x 10⁵ trophozoites or cell extract were added in 800 µl of a serum-free mixture of RPMI and TYI-S-33 medium (5:3) to the cells. The amount of CHO cells that remained attached was proportional to the intensity of methylene blue staining. The amount of dye extracted from CHO monolayers that had not interacted with trophozoites served as controls (0% destruction of cell monolayer). Experiments were carried out at least in triplicate and were repeated twice.

Transmission electron microscopy—For electron microscopy the trophozoites were first cryoimmobilized using the procedure of high-pressure freezing of cell
suspensions as described by Hohenberg et al. (36). After high-pressure freezing the frozen capillary tubes were freed from extraneous 1-hexadecene under liquid nitrogen. The samples were then transferred into precooled test tubes, filled with acetone containing 0.5% uranyl acid. Dehydration was carried out in a conventional freeze-solution unit (AFS, Leica Bensheim, Germany) at –90°C for 8 h, followed by two substitution steps at –70°C and –50°C for 8 h. After freeze substitution the samples were infiltrated with Epon 812 (Fluka, Germany). The samples were washed in pure acetone for 30 min at –50°C, infiltrated with 30% (v/v) Epon and 70% (v/v) acetone for 2 h at 4°C, then infiltrated with 70% Epon and 30% acetone for 2 h at 4°C, before finally four incubation steps were performed for 2 h each in pure Epon at RT. Polymerization was carried out at 60°C for 24 h. Ultrathin sections contrasted with uranylacetate and lead citrate observed with a Zeiss EM 910 transmission electron microscope.

**Two-dimensional gel electrophoresis, in-gel digestion of proteins and mass spectrometry**—Two-dimensional (2-D) gel electrophoresis, in-gel digestion, matrix-assisted laser desorption/ionization mass spectrometry (MALDI TOF MS) and database analysis was performed as previously described (37). For each 2-D gel 500 μg of soluble cell extract was used as sample.

**Induction of amoebic liver abscesses in gerbils**—Gerbils were challenged with the direct hepatic inoculation of 1 x 10^6 E. histolytica trophozoites according to the previously described methods (38). Seven days later, animals were sacrificed, the liver was entirely removed and sectioned, and the weight of abscesses relative to the total liver weight was determined.
RESULTS

_Growth of E. histolytica trophozoites in the presence of cysteine proteinase inhibitors_—In order to determine the growth characteristics of _E. histolytica_ trophozoites in response to various cysteine proteinase inhibitors (CPI), amoebae were cultivated in the presence of different amounts of E64, E64d, Z-Phe-Ala-DMK, Z-Phe-Phe-DMK, and HMB, respectively. In contrast to the Z-Phe-Phe-DMK, which had almost no effect on amoeba growth, all other compounds inhibited growth in a dose-dependent manner (Fig. 1A). For HMB as well as the membrane-permeable CPIs E64d and Z-Phe-Ala-DMK 10 - 20 µM were sufficient to kill all cells within 72 h. The non membrane-permeable CPI E64 was less potent as higher amounts of the drug were necessary to kill the cells. In the presence of 100 µM of E64 nearly all amoebae died within 3 days (Fig. 1A). However, a small proportion of about 0.1% of the cells was found to be resistant to this inhibitor. Resistant cells regularly multiplied and were able to survive in the presence of as much as 500 µM of E64, although the doubling time decreased by about 50%. In the absence of E64, resistant cells revealed a similar growth curve as unselected wild-type amoebae. Resistance to E64 was a stable property, as culturing in the absence of E64 for up to at least 4 months did not substantially affect subsequent growth in the presence of the drug. Re-addition of E64 revealed only a short delay of about 2 to 3 days until the cells started to multiply regularly and entering logarithmic growth phase (Fig. 1B). Interestingly, E64-resistance was not associated with resistance to other CPIs. Sensitivity to HMB, E64d or Z-Phe-Ala-DMK was identical between E64-resistant and wild-type amoebae (Fig. 1A). Moreover, various attempts to generate _E. histolytica_ trophozoites resistant to E64d, Z-Phe-Ala-DMK or HMB were unsuccessful.
Comparison of cysteine proteinase expression in E64-sensitive and E64-resistant *E. histolytica*—Total CP activity in trophozoite lysates as determined by cleavage of the synthetic substrate Z-Arg-Arg-pNA was found to be identical in wild-type and E64-resistant cells. In the absence of E64, CP activities were about 200 mU/mg, whereas short-term cultivation for a few hours in the presence of 50 µM of E64 completely blocked CP activity in both wild-type and resistant cells. Time-course experiments after removal of E64 from the culture revealed a constant increase of CP activity with full recovery after 72 h as determined by substrate gel electrophoresis (Fig. 2).

Previous studies have shown that under standard culture conditions *E. histolytica* trophozoites express three major CPs, namely EhCP1, EhCP2 and EhCP5, respectively, which all run in SDS polyacrylamide gels under reduced conditions as bands of approximately 29 kDa (33). Western blot analysis using antibodies specific to the various CPs revealed that cultivation in the presence of E64 increases the molecular mass by approximately 5 kDa (Fig. 3). This was seen in E64-resistant as well as in wild-type amoebae. Separation of the higher molecular mass CPs by two-dimensional gel electrophoresis and subsequent analysis of isolated proteins by MALDI TOF MS indicated that the three proteins represent unprocessed proforms of EhCP1, EhCP2 or EhCP5 (data not shown).

In addition to protein analysis, RNA expression of the corresponding *E. histolytica* cp-genes was compared between wild-type and resistant amoebae cultivated in the presence or absence of E64. E64-sensitive wild-type cells were cultivated in the presence of 50 µM of E64 for only 24 h, whereas E64-resistant amoebae were grown with the inhibitor for 7 days. Northern blot analysis indicated substantial more RNA of the various *E. histolytica* cp-genes in E64 sensitive cells irrespectively whether grown in the presence or absence of E64. However, both
resistant and sensitive amoebae showed an increase of \( cp1 \)- and \( cp2 \)-RNA when grown in the presence of E64, whereas \( cp5 \)-RNA increased only in wild-type cells (Fig. 4).

\[ \text{Secretion of cysteine proteinases} \]

It has been previously demonstrated that \( E. \ histolytica \) trophozoites secrete cysteine proteinases into the culture medium (34). However, the amount of enzyme is limited and can be detected only by the use of sensitive activity assays but not by Western blots. However, when we developed Western blots from culture supernatants of E64-resistant cells, grown in the presence of E64, a specific band was found to react with anti-CP antibodies. This band corresponded to the band of unprocessed CPs in amoeba extracts (Fig. 5). In contrast, this band was not detected in culture supernatants of wild-type cells grown in the presence of E64 or in wild-type and E64-resistant cells grown without E64. The result suggests that the E64-resistant phenotype is able to secrete substantial amounts of unprocessed cysteine proteinases.

\[ \text{Ultrastructure of} \ E. \ histolytica \ \text{cultivated in the presence of E64} \]

E64-resistant and sensitive amoebae were subjected to electron microscopy for ultrastructural analysis. In the presence of E64 both types of cells showed a massive network of fibrillae-like structures located in the cytoplasm, which was not observed in trophozoites cultivated in the absence of E64 (Fig. 6). These structures were obviously devoid of actin, as they did not react with antibodies, which were able to detect \( E. \ histolytica \) G- or F-actin (data not shown).

\[ \text{E64 and cytopathic activity} \]

\( E. \ histolytica \) trophozoites or trophozoite lysates are able to disrupt monolayers of cultured mammalian cells such as Chinese
hamster ovary (CHO) cells. This phenomenon, called the cytopathic effect, can be blocked by preincubation with E64 (39). Comparison between wild-type and E64-resistant amoebae in the presence or absence of E64 revealed no differences in cytopathic activity (Fig. 7A). In the absence of E64 both types of cells disrupted about 75% of a CHO cell monolayer within 1 h, whereas preincubation of amoeba with E64 reduced the cytopathic effect to about 15%. Similar results were obtained with corresponding trophozoite lysates (Fig. 7B).

Amoebic liver abscess formation in the presence of E64—Previous studies have shown that *E. histolytica* cysteine proteinases play a major role for the development of amoeba-induced liver abscesses in experimentally infected rodents. Accordingly, we have analyzed whether preincubation of amoeba with E64 or E64-resistance has any influence on abscess formation. Groups of gerbils consisting of 8 animals each were challenged either with wild-type amoebae cultured for 24 h or with E64-resistant cells cultivated for 7 days in the presence of 50 µM of E64. As controls, groups of animals were challenged either with wild-type or with E64-resistant amoebae cultured without E64. Wild-type and E64-resistant controls induced liver abscesses in 8 out of 8 and 5 out of 8 gerbils, respectively, whereas the same cells cultured in the presence of E64 revealed abscess formation in 6 out of 8 and 1 out of 8 animals, respectively. Thus, only the group of gerbils, which was infected with E64-resistant amoebae, cultured in the presence of E64, had significant fewer abscesses (*P* < 0.02). However, compared to the wild-type control group, sizes of abscesses in animals, which were not completely protected, were significantly smaller when challenged with E64-resistant cells (*P* < 0.05) or with wild-type cells cultured in the presence of E64 (*P* < 0.001) (Table 1).
Discussion

Cysteine proteinases have been shown to constitute major pathogenicity factors of *E. histolytica* and are considered as suitable targets for antiparasitic drugs. However, present knowledge regarding the significance of the various CPs for *E. histolytica* growth, pathogenicity or the parasite life-cycle is still fragmentary. In an attempt to further elucidate the importance of CPs, we have grown *E. histolytica* trophozoites in the presence of different inhibitors. The results clearly demonstrate that broad-range CPIs, which rapidly enter the cell, are deleterious for the parasite at least under axenic culture conditions. In contrast, one membrane-permeable cysteine proteinase inhibitor Z-Phe-Phe-DMK has nearly no effect on *E. histolytica* growth. Like the potent amoebic growth inhibitor Z-Phe-Ala-DMK, Z-Phe-Phe-DMK belongs to the diazomethylketones, which are described as potent CPIs. It is also described that Z-Phe-Ala-DMK was a much more potent inhibitor for Cathepsin L and B like enzymes in comparison to Z-Phe-Phe-DMK. In contrast, Z-Phe-Phe-DMK is a very poor inhibitor of Cathepsin B but is a better inhibitor of Cathepsin L than is Z-Phe-Ala-DMK (40,41). *In vitro* studies using amoebic extracts confirm these data. The main expressed proteinases EhCP1, EhCP2 and EhCP5 exhibit Cathepsin B like activity (32,42,43), and our results indicate that Z-Phe-Ala-DMK is a 50-fold more potent inhibitor than is Z-Phe-Phe-DMK (data not shown). Therefore it is no surprise that Z-Phe-Ala-DMK has an influence on the growth of *E. histolytica*, whereas the Z-Phe-Phe-DMK has not. Moreover, all attempts to select amoebae resistant to these highly potent inhibitors were unsuccessful indicating the importance of CPs for parasite growth and further supporting the concept of the use of CPs as targets for the development of antiamoebic drugs. However, it remains to be determined whether indeed a large number of the various *E. histolytica* CPs or only a few or a specific
subset have to be targeted to inhibit amoeba growth. At least downregulation of EhCP5 as previously performed by anti-sense technology, which resulted in a 90% reduction of total CP activity, did not affect amoeba growth in vitro but cells were impaired in their ability to induce amoebic liver abscess in rodents (24).

In contrast to the various highly potent compounds including E64d, E64 was less toxic and inhibited amoeba growth only at 10-fold higher concentrations. Compared to E64d, E64 has the same substrate specificity, but does not effectively penetrate membranes (44). However, E64 can be taken up by the parasite via pinocytosis. Thus, it may primarily act against surface bound CPs as well as against enzymes located in phagolysosomes, but may be less effective against CPs that are active at other destinations within the cell including those involved in protein trimming and maturation processes. The latter function of CPs is likely to be of critical importance for amoeba growth and may explain the high toxicity of CPIs that rapidly penetrate membranes.

Another interesting result of this study was the finding that E. histolytica trophozoites are able to counteract the toxic effect of E64 by developing resistance to this compound. E64-resistance was a stable property and was maintained even after growing of the parasite for several months in the absence of the drug. Thus, the mechanism of resistance differs from that recently reported for metronidazole, as in vitro-induced resistance of E. histolytica to metronidazole was rapidly lost after a few passages (26,27).

A number of different mechanisms responsible for drug resistance in protozoan parasites have been identified in recent years (45-48), including those decreasing the influx or accelerating the efflux of therapeutic agents. Although we do not know at present the mechanism that is operational for E64-resistance in Entamoeba, it is less likely that the reduction of the drug load is of importance, as we
did not find any differences in time-course and degree of CP inhibition by E64 between wild-type and resistant cells.

The mode of action of CPIs has been studied in considerable detail in *Trypanosoma cruzi*. It was suggested that the lethal effect of CPIs to this protozoan parasite was due to interruption of the autocatalytic processing of proteinases, leading to their accumulation in the Golgi complex and subsequent disruption of trafficking and processing of glycoproteins in the parasite (14). *Trypanosoma cruzi* made resistant to CPIs showed upregulation of the secretory pathway, and ultrastructural studies revealed a marked increase in the number of vesicles trafficking from the Golgi complex to the flagellar pocket, the side of protein excretion in trypanosomes. Moreover, in resistant cells, the main cysteine proteinase, cruzain, which is usually targeted to lysosomes, was found to be localized within the lumen of and in vesicles proximal to the flagellar pocket and was secreted as unprocessed, inactive proform (49). Likewise, autocatalytic processing of CPs was inhibited in *E. histolytica* trophozoites challenged with E64 and E64-resistant amoebae were found to secrete substantial amounts of unprocessed enzyme. On the other hand, E64-resistance of *E. histolytica* differed in several aspects from CPI-resistance of *T. cruzi*. E64-resistant amoebae could be rapidly selected from standard cultures but revealed no cross-resistance to other CPIs. In contrast, induction of resistance in *T. cruzi* required more than one year of selection increasing the drug step-wise and resistant cells were cross-resistant to at least another 4 different CPIs (49). Moreover, ultrastructural analysis of E64-resistant amoebae did not reveal a marked increase in the contents of vesicle. However, *Entamoeba* trophozoites contain an extraordinary high number of vesicles. Thus, more detailed studies are required until a final conclusion can be made whether the number of excretory vesicles has been changed in resistant cells. The only obvious ultrastructural change that became
visible upon challenge with E64 was the occurrence of a massive network of fibrillae-like structures located in the cytoplasm of the cells. These structures did not contain actin but may consist of tubulin and might constitute as response to stress as they were found not only in E64-resistant and but also in E64-sensitive amoebae when grown in the presence of the inhibitor. One of the main differences between wild-type and E64-resistant \textit{E. histolytica} was a substantial impairment of resistant amoebae to induce liver abscesses in laboratory animals. In agreement with previous findings (21), pre-incubation of sensitive cells with E64 did not completely block liver abscess formation but compared to respective controls abscesses were significantly smaller. In contrast, sizes of abscesses were significantly reduced in E64-resistant cells even in the absence of E64 and liver abscess formation was completely blocked in 7 out of 8 animals when infected with E64-resistant amoebae grown in the presence of the inhibitor. The reduced virulence of E64 resistant amoebae might be linked to the observed decreased expression of the various \textit{ehcp} genes (Fig. 4). In particular the strong reduction of \textit{ehcp5}-RNA together with a loss of a regular response in the presence of E64 might be of importance. Increasing evidence suggests that EhCP5 plays a critically role for \textit{E. histolytica} pathogenicity. The molecule is present on the surface of the amoeba and activates inflammatory cytokines (25,32). Interestingly, a corresponding enzyme is absent in the closely related, nonpathogenic species \textit{Entamoeba dispar} (50,51). Moreover, \textit{E. histolytica} with downregulated EhCP5 by \textit{ehcp5} antisense-RNA were unable to induce liver abscesses in hamsters and showed less gut inflammation in mice (24,25).
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Figure legends

Figure 1

**Growth curves of WT and E64r amoebae.** A. WT and E64r amoebae (1000 amoebae/well) were cultivated for 72 h in the presence of different CPIs as indicated. The inhibition of growth, as % to the respective control (cultivation without inhibitor) was determined. ● WT amoebae, □ E64r amoebae.

B. WT as well as E64r amoebae (2000 amoebae/well) were cultivated in the absence or presence of E64 (100 µM). The number of viable cells was determined as described in material and methods. Each experiment was done two times in triplicate.

● WT + E64, □ E64r + E64, ○ WT, □ E64r.

Figure 2

**Enzymatic activity of WT and E64r amoebae cultivated in the presence or absence of E64.** E64r amoebae were cultivated for 7 days in the presence of E64 (50 µM). Subsequently E64 was removed from the medium and the amoebae were cultivated for additional 8 days in the absence of E64. After different time points cells were harvested and the respective extracts were analyzed for CP activity using substrate gel electrophoresis and CP activity assay. WT amoebae cultivated for 24 h in the presence of E64 were used as control.

Note: Previous findings indicate that the 35 and 48 kDa proteins correspond to EhCP2 and EhCP1, respectively (52). So far, it is not known which of the amoebic CPs are responsible for the other activity bands.
Figure 3

**Western blot analysis of total extracts from WT and E64r amoebae cultivated in the presence and absence of E64.** Following separation on a 12% SDS-polyacrylamide gel, proteins were transferred to nitrocellulose, and the blot was developed with antibodies specific for the respective CPs as indicated. All samples were incubated with 50 mM DTT for 5 min at 95°C with the exception of anti-EhCP5 in which all samples had to be reduced at 37°C. Standards are indicated in kDa at the left.

Figure 4

**Northern blot analysis of WT and E64r amoebae cultivated in the absence or presence of 50 µM E64 (24 h and 7 days, respectively).** Total RNA of the respective amoebae was extracted, electrophorized on agarose gels, blotted and sequentially hybridized with the genes encoding *ehcp1*, *ehcp2*, *ehcp3* and *E. histolytica* actin.

Figure 5

**Western Blot analysis of total extracts and culture supernatants from WT and E64r amoebae cultivated in the presence or absence of E64.** Following separation on a 12% SDS polyacrylamide gel, proteins were transferred to nitrocellulose, and the blot was developed with an antibodies against amoebic CP. All samples were incubated with 50 mM DTT for 5 min at 95°C. Standards are indicated in kDa at the left.
Figure 6

Transmission electron micrograph of cryofixed and cyrosubstituted WT and E64r amoebae.

Figure 7

Cytophatic activity. CHO cells (1 x 10^5) were grown to confluence in 24-well plates. After washing of the CHO cells with phosphate-buffered saline, 1 x 10^5 trophozoites (A) or cell extract (200 µg) (B) were added in 800 µl of a serum-free mixture of RPMI and TYI-S-33 medium (5:3) to the cells. The amount of CHO cells remaining attached was proportional to methylene blue staining. The destruction of cell monolayer (%) was determined.
Table 1. Liver abscess formation in gerbils challenged with *Entamoeba histolytica*

| Amoebic population | No. of gerbils with abscess / No. of gerbils challenged | Percent liver abscessed in nonprotected gerbils |
|---------------------|--------------------------------------------------------|-----------------------------------------------|
| WT                  | 8 / 8                                                   | 7.53±3.65                                     |
| WT+E64\(^1\)        | 6 / 8                                                   | 1.82±1.07 (\(P < 0.001\))\(^4\)*            |
| E64r                | 5 / 8                                                   | 2.96±2.25 (\(P < 0.05\))\(^4\)*            |
| E64r+E64\(^2\)      | 1 / 8 (\(P < 0.02\))\(^3\)*                           | 1.38                                          |

\(^1\)cultured in the presence of 50 µM of E64 for 24 hr; \(^2\)cultured in the presence of 50 µM of E64 for 7d; \(^3\)compared to WT+E64; \(^4\)compared to WT; *\(P\) values were determined by paired t-test
Figure 1
Figure 2
Figure 3
Figure 4
Nowak et al., E64-resistance of *E. histolytica*

![Figure 5](image_url)

**Figure 5**

| kDa | R | R | C | C | R | R | C | C | R | R | C | C |
|-----|---|---|---|---|---|---|---|---|---|---|---|---|
| 46  |   |   |   |   |   |   |   |   |   |   |   | + |
| 35  |   |   |   |   |   |   |   |   |   |   |   | + |
| 24  |   |   |   |   |   |   |   |   |   |   |   | + |
| 17  |   |   |   |   |   |   |   |   |   |   |   | + |

E64 (50 μM)

Trophozoites  Culture supernatant
Figure 6
Figure 7
Resistance of entamoeba histolytica to the cysteine proteinase inhibitor E64 is associated with secretion of pro-enzymes and reduced pathogenicity
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