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Major cysteine peptidases of *Entamoeba histolytica* are required for aggregation and digestion of erythrocytes but are dispensable for phagocytosis and cytopathogenicity

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Summary

Cysteine peptidases of *Entamoeba histolytica* (EhCPs) are considered to be important pathogenicity factors. It has been described that under standard axenic culture conditions, only three (*ehcp-a1*, *ehcp-a2* and *ehcp-a5*) out of approximately 50 cysteine peptidase genes present in the *E. histolytica* genome are substantially expressed, thus representing the set of major EhCPs. In this study, transcriptional silencing of the major peptidase genes was used to characterize their physiological role in more detail. Analysing the transfectants a fourth major cysteine peptidase activity belonging to EhCP-A7 could be characterized. Neither cytopathic activity nor phagocytosis of erythrocytes was altered in CP-inactivated amoebae. However, a significant difference in haemolytic activity was observed. EhCP-A1 and EhCP-A7 apparently had no influence on haemolytic activity, whereas transfectants silenced for *ehcp-a5* as well as those silenced for all major peptidases showed a significant reduction in their haemolytic activity. Furthermore, cells silenced for *ehcp-a1* and *ehcp-a7* and more effectively cells silenced in all major *ehcp*s were impaired in digesting of phagocytosed erythrocytes. Moreover, amoebae silenced for all major peptidase genes lost the ability to form aggregates of erythrocytes prior to phagocytosis.

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Introduction

The faecal–oral spread protozoan parasite *Entamoeba histolytica* is an important pathogen, responsible for millions cases of invasive amoebiasis each year (WHO, 1997). *E. histolytica* usually resides in the large bowel where it can persist for months or even years causing asymptomatic luminal gut infections. However, occasionally *E. histolytica* penetrates the intestinal mucosa and destroys host tissues. Parasite invasion leads to ulcerative colitis or dissemination to other organs, most commonly to the liver, and may result in abscess formation.

Identification of factors that are responsible for the pathogenicity of *E. histolytica* has been a major topic in the field. During recent years, cysteine peptidases (CPs) have been identified to play an important role for the pathogenicity of *E. histolytica* as suggested by a large number of *in vitro* and *in vivo* studies (Gadasi and Kessler, 1983; Lushbaugh *et al*., 1985; Luaces and Barrett, 1988; Reed *et al*., 1989; Schulte and Scholze, 1989; Keene *et al*., 1990; Li *et al*., 1995; Stanley *et al*., 1995). Most convincing are results from infections of laboratory animals indicating that *E. histolytica* trophozoites that have reduced CP activity are greatly impaired in their ability to induce amoebic disease (Stanley *et al*., 1995; Ankri *et al*., 1999). In addition, overexpression of CPs increases cytopathic activity of the parasite, as measured by *in vitro* monolayer disruption, and induces more severe amoebic liver abscesses in laboratory animals (Tillack *et al*., 2006). Other studies suggested that CPs are involved in phagocytosis, as amoebae with a reduced CP activity revealed significantly reduced phagocytotic activity (Ankri *et al*., 1998; Hirata *et al*., 2007). So far, out of the 50 *cp* genes present in the *E. histolytica* genome, only three (*ehcp-a1*, *ehcp-a2* and *ehcp-a5*) have been described to be expressed in substantial amounts in amoeba trophozoites under standard axenic culture conditions (Bruchhaus *et al*., 2003; Clark *et al*., 2007; Tillack *et al*., 2007). Accordingly, most studies on *E. histolytica* CPs as well as all biochemical characterizations of the enzymes were performed with EhCP-A1, EhCP-A2 and EhCP-A5.

To further analyse the biological function of the three major peptidases we silenced their expression in

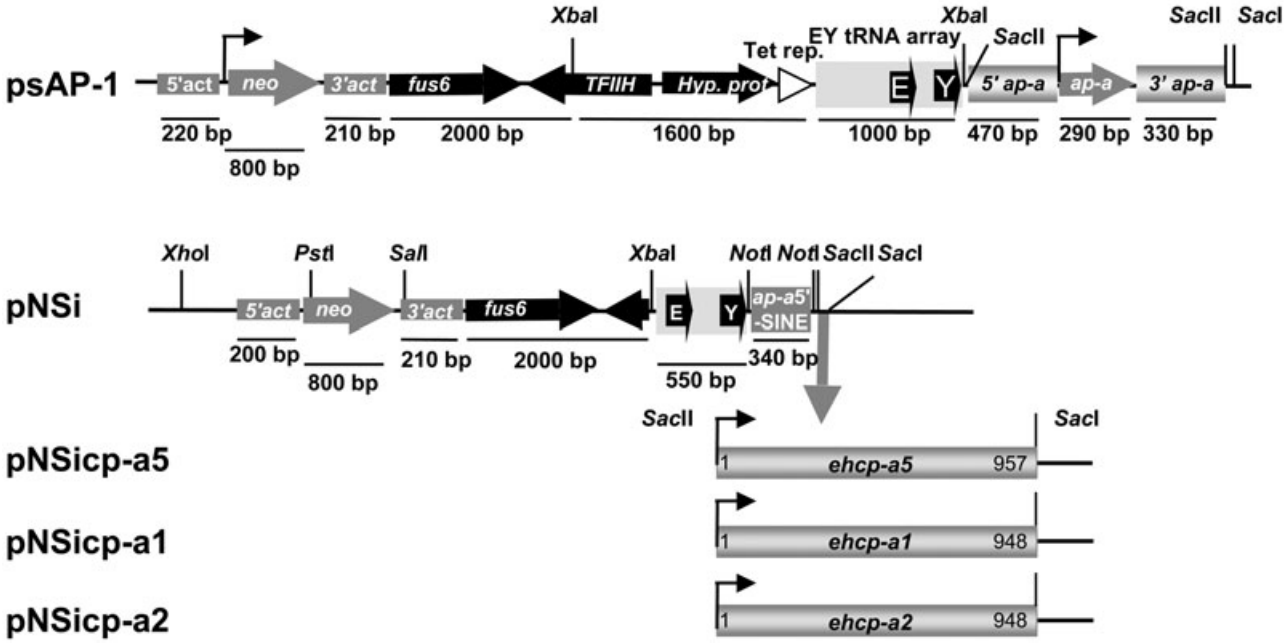

Fig. 1. Schematic depiction of plasmid vectors used to silence the expression of cysteine peptidase genes. The plasmid pNSi is a derivative of psAP1 published by Bracha *et al.* (2003). The gene of interest was inserted into the SacII and SacI restriction site of the pNSi vector.

E. histolytica using a derivative of a silencing approach recently described by Bracha *et al.* (2003). At present, this method is the only way to reproducibly inactivate expression of a gene of interest in *E. histolytica*. Although this approach has inherent limitations, it allows to study the physiological relevance of various genes for this organism (Bracha *et al.*, 2006; Mirelman *et al.*, 2008).

In this study, we generated transgenic amoebae transcriptionally silenced for various major *ehcp* genes or for combinations of them. These transgenic amoebae allowed the identification of a fourth major peptidase activity in substrate gels. Furthermore we have shown that formation of erythrocyte aggregates as well as haemolysis and digestion of erythrocytes depends on the activity of these peptidases, whereas phagocytosis and cytopathogenicity do not.

Results

Generation of transgenic amoebae silenced for different CP genes

Silencing of gene expression has been achieved in a specific *E. histolytica* isolate termed HM-1:IMSS:2411 (Bracha *et al.*, 2003; Mirelman *et al.*, 2008). In a first step, the gene for the pore-forming protein amoebapore A (*ehap-a*) has to be silenced using the vector psAP1 (G3-trophozoites). Subsequently, these cells can be used to silence the expression of additional genes. In this study, we used a derivative of psAP1 for silencing of various *ehcp* genes. This vector, which was termed pNSi, contains a smaller 5′ *ehap-a* region (350 bp) and a region of approximately 2600 bp containing genes that are not important for silencing was deleted (Fig. 1). Coding sequences of *ehcp-a1*, *ehcp-a2* or *ehcp-a5* were cloned into pNSi and transfected into G3 plasmid-less trophozoites.

Real-time PCR as well as microarray analyses indicated that the expression profiles of HM-1:IMSS and G3-trophozoites are very similar to each other (Tillack *et al.*, 2007). In HM-1:IMSS as well as in G3-trophozoites, the three most abundant *ehcp* genes are *ehcp-a2*, *ehcp-a1* and *ehcp-a7* followed by *ehcp-a5*. All other *ehcp* genes were found to be expressed at very low levels (Table 1). It has to be kept in mind that a relative expression to *ehactin* (ΔCT; used as normalizer) > 4.0 is below the detection limit of the microarrays used in our studies (Tillack *et al.*, 2007 *).*

After transfection of G3-trophozoites with the plasmid pNSicp-a1 two clones were obtained with different expression properties. In clone 1 (pNSicp-a1:C1-transfectants) only *ehcp-a1* was silenced, whereas in clone 2 (pNSicp-a1:C2-transfectants) *ehcp-a1* and *ehcp-a7* (79% identity) were silenced. Expression of other *ehcp* genes was not influenced (Table 1). Cells transfected with pNSicp-a5 (pNSicp-a5-transfectants) revealed an almost complete reduction in *ehcp-a5* expression. In addition, for *ehcp-a4*, *ehcp-a6* and *ehcp-a8*, which are already expressed at very low levels in *E. histolytica* and which have a high sequence similarity to *ehcp-a5* (63–

Table 1. Expression of *ehcp* genes in various transfectants analysed by real-time PCR.

Cysteine peptidase gene (target)	ΔCT[a] HM-1:IMSS	ΔCT G3	G3 (calibrator)	Relative concentration (ΔΔCT method) Transfectants			
				pNSicp-a1:C1	pNSicp-a1:C2	pNSicp-a5	pNSicp-a2
ehcp-a2	−0.81	−1.34	1	1.6	1.6	2	0[b]
ehcp-a1	−0.70	−0.68	1	0.01[b]	0.01[b]	1.1	0.01[b]
ehcp-a7	2.51	1.10	1	1.3	0[b]	1.7	0[b]
ehcp-a5	2.61	1.28	1	1.8	1	0.01[b]	0[b]
ehcp-a4	5.82	5.88	1	0.8	0.7	0.01[b]	0[b]
ehcp-a6	7.53	6.25	1	0.6	1	0.01[b]	0[b]
ehcp-a8	9.56	9.20	1	1.2	1	0[b]	0.04[b]
ehcp-a3	12.38	11.56	1	0.5	1.1	1	2
ehcp-b4	5.79	7.14	1	1.6	1.6	1.6	1.3
ehcp-b8	18.42	7.69	1	1.9	1.5	1.3	1.8
ehcp-b3	11.97	9.09	1	1	1	1	0.8
ehcp-b5	9.90	9.10	1	0.9	0.8	0.8	0.9
ehcp-b9	10.30	9.33	1	1.1	1.1	1.3	0.9
ehcp-b7	8.22	10.56	1	1.4	1.3	1.5	1
ehcp-b1	14.17	11.63	1	2	0.7	3	2
ehcp-c4	4.25	3.77	1	1.1	0.9	1.3	1.3
ehcp-c5	4.93	3.68	1	1.1	1.1	1.3	1.7
ehcp-c8	7.58	4.67	1	1.5	1.4	1.6	2
ehcp-c1	9.64	8.72	1	0.8	1	1.1	1

a. Relative concentration to ehactin, which was used as normalizer.
b. Expression of the respective gene is silenced.

68% identity) no transcripts were detected. Interestingly, several attempts failed to generate *ehcp-a2*-silenced cells by transfecting G3-trophozoites with pNSicp-a2 (pNSicp-a2-transfectants). However, this approach resulted in a single clone that was silenced not only for *ehcp-a2*, but also for *ehcp-a1*, *ehcp-a4*, *ehcp-a5*, *ehcp-a6*, *ehcp-a7* and *ehcp-a8* respectively. The expression of *ehcp-a3* or other members of the *ehcp* families B and C was not influenced in this clone, as revealed by microarray analysis or real-time PCR (Table 1). This transfectant showed a significantly reduced growth rate.

Substrate gel electrophoresis

The analysis of the various transfectants using substrate gel electrophoresis indicated a good correlation between the results of transcription and peptidase activities. Moreover, this analysis allowed the assignment of the various enzymes to bands of particular sizes (Fig. 2). Although the calculated molecular masses of the mature form of the four major peptidases are very similar (approximately 24 kDa) the migration behaviour in substrate gel varies substantially (Hellberg *et al*., 2000; 2001; Tillack *et al*.,

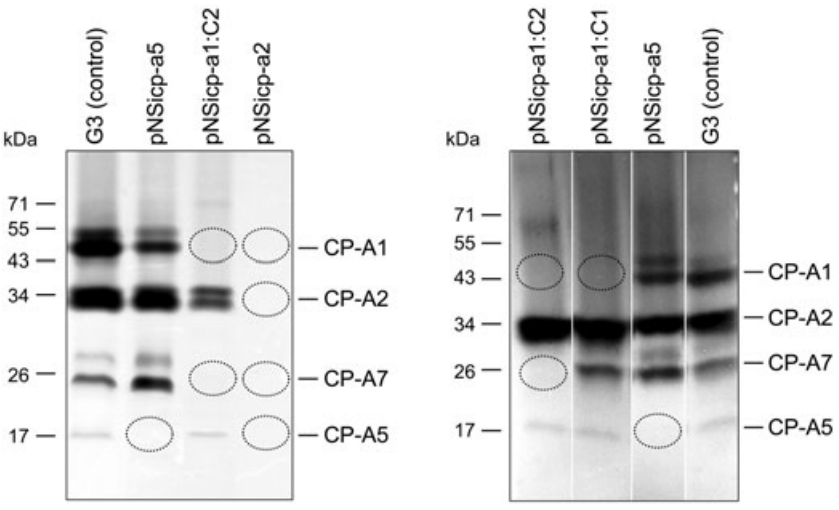

Fig. 2. Substrate gel electrophoresis of transgenic amoebae silenced for different cysteine peptidases (CPs). G3-trophozoites were transfected with episomal plasmids pNSicp-a5, pNSicp-a1 and pNSicp-a2 as indicated and selected with G418. Subsequently, lysates of the cells were separated on SDS-PAGE co-polymerized with gelatine. To visualize the CP activity of proteins, gels were stained with Coomassie blue and the picture was inverted. Standards are indicated in kDa on the left.

Table 2. Specific activity, rate of erythrophagocytosis and rate of digestion of erythrocytes of the various *cp*-silencing transfectants.

Transfectants	Specific activity (mU mg^{-1})[a]	Rate of erythrophagocytosis[b]	Rate of digestion of erythrocytes[c]
G3 (control)	91 ± 6	57.8 ± 8.6	69.0 ± 7.5
pNSicp-a5	92 ± 5	70.8 ± 6.0	53.6 ± 3.3
pNSicp-a1:C1	74 ± 5	54.2 ± 10.3	53.3 ± 12.4
pNSicp-a1:C2	59 ± 3	60.8 ± 9.4	25.8 ± 6.9**
pNSicp-a2	1 ± 1	60.0 ± 9.2	6.7 ± 2.5***

a. The specific activity (mU mg^{-1}) was measured using the synthetic substrate Z-Arg-Arg-pNA.
b. After 1 h of incubation in the presence of erythrocytes the non-phagocytosed erythrocytes were lysed and the percentage amoebae containing more than five erythrocytes per amoebae were determined.
c. After 2 h of digestion of erythrocytes the percentage amoebae containing no erythrocytes/amoebae were determined.
***$P < 0.001$, **$P < 0.01$.

2006). The reason for this disparate migration behaviour is not clear yet. Nevertheless, the results were in agreement with previous findings indicating that activity bands of 48 and 34 kDa most likely correspond to EhCP1 and EhCP2 respectively (Hellberg *et al.*, 2000; 2001). In addition, an activity band at a molecular mass of 26 kDa could be assigned to EhCP-A7 and the band at 17 kDa to EhCP-A5.

CP activity against the synthetic substrate Z-Arg-Arg-pNA

The specific CP activity of G3-trophozoites measured using the synthetic substrate Z-Arg-Arg-pNA was 91 ± 6 mU mg^{-1}. Silencing of *ehcp-a5* only had no implication on the total proteolytic activity (92 ± 5 mU mg^{-1}). Silencing of *ehcp-a1* however resulted in a 20% reduction of total CP activity (74 ± 5 mU mg^{-1}) whereas the combined silencing of *ehcp-a1* and *ehcp-a7* reduced total CP activity by about 35% (59 ± 3 mU mg^{-1}), and for pNSicp-a2-transfectants an almost complete loss of EhCP activity was observed (1 ± 1 mU mg^{-1}; Table 2). These results suggest that the majority of CP activity from amoebic lysates is attributed to EhCP-A2, followed by EhCP-A1 and EhCP-A7, whereas the portion of EhCP5 is negligible for total activity in the analysed protein fraction. The order of activity is consistent with the picture seen in substrate gel electrophoresis as well as with the expression profile analysed using real-time PCR or microarray analysis.

Cytopathic and haemolytic activity of E. histolytica *transfectants*

Previous studies have shown that viable *E. histolytica* trophozoites or amoebic extracts are able to disrupt monolayers of cultured mammalian cells, such as Chinese hamster ovary (CHO) cells, and that EhCPs may be involved in this cytopathic process. In addition, it has already been shown that G3-trophozoites are impaired in their ability to destruct monolayers of BHK cells (Bracha *et al.*, 2003). Bracha and colleagues showed that after 1 h incubation of G3-trophozoites with BHK cells only 10% of the monolayer is disrupted, whereas the respective controls showed a 90% disruption. Under the experimental conditions used in this study (CHO cells instead of BHK cells and an incubation period of 90 min) control G3-trophozoites showed also a reduction in their cytopathic activity. Whereas HM-1:IMSS:2411 trophozoites were found to disrupt approximately 90%, G3-trophozoites disrupt only 40% of a CHO monolayer. However, additional silencing of the various *ehcp* genes did not lead to a further reduction in cytopathic activity. All transfectants analysed showed also an approximately 40% disruption of the cell monolayer (data not shown).

In contrast, significant differences in the haemolytic activity were observed between G3-trophozoites and pNSicp-a5- as well as pNSicp-a2-transfectants. Both pNSicp-a5- and pNSicp-a2-transfectants revealed a reduction in haemolytic activity by 39% and 48% respectively. On the other hand, transfectants in which the gene for EhCP-A1 or EhCP-A1 and EhCP-A7 were silenced did not show any reduction in haemolytic activity (Fig. 3).

Phagocytosis and digestion of human erythrocytes

Previous studies have suggested that EhCPs are involved in phagocytosis. Therefore, the uptake of erythrocytes

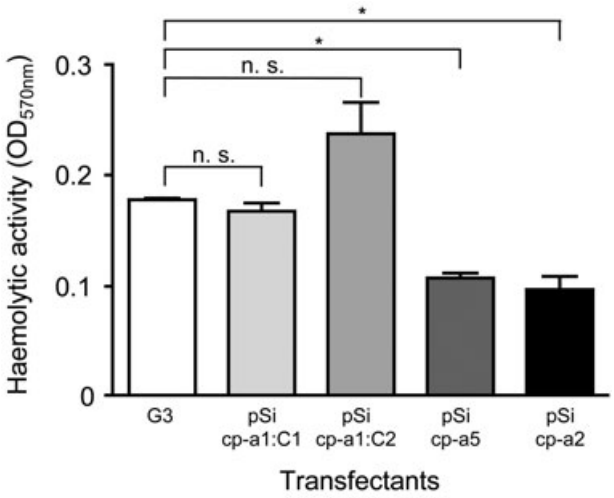

Fig. 3. The assay to determine the haemolytic activity was performed by mixing the respective transfectants and erythrocytes in a ratio of 1:2000 followed by incubation for 1 h at 37°C. After incubation the cells were sedimented and haemoglobin that was released in the supernatant was read at 570 nm in a spectrophotometer. n.s., not significant. *$P < 0.03$.

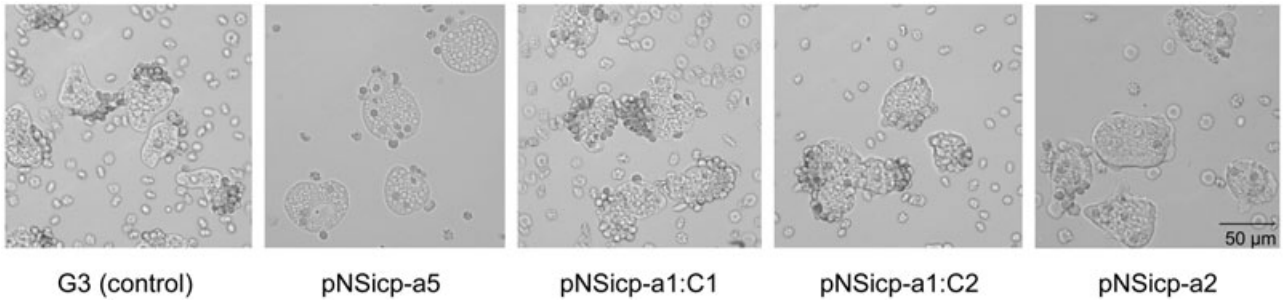

Fig. 4. To analyse the aggregation of erythrocytes prior to phagocytosis the respective transfectants were mixed with erythrocytes, applied onto a slide and erythrocyte binding was detected directly by light microscopy.

was analysed in the various transfectants. However, no differences were observed between them. All cell lines had the same capacity of erythrophagocytosis similar to that of the respective control cells (Table 2). In contrast, major differences were observed concerning the rate of erythrocyte digestion. Two hours post phagocytosis, 70% of the control cells had completely digested the phagocytosed erythrocytes, whereas digestion rate was reduced in the various *ehcp*-silenced transfectants and directly correlated with the level of EhCP activity. In amoebae silenced for *ehcp-a1* or *ehcp-a5*, digestion was complete in 53% of the cells, whereas in parasites silenced for *ehcp-a1* and *ehcp-a7* or for all major *ehcp* genes complete digestion of erythrocytes was found in only 26% and 7% of cells respectively (Table 2). It is already known that not all cells in an amoebic population are able to phagocytose. This may be attributed to the developmental stage of the individual cell. In the cell populations analysed in this study, between 3% and 14% of a population are unable to phagocytose (data not shown). Therefore, it can be concluded that the 7% cells found in pNSicp-a2-transfectants that did not contain erythrocytes (Table 2) after 2 h of incubation are cells that have not phagocytosed and consequently silencing of all *ehcp* genes led to a total inhibition of digestion.

Formation of erythrocyte aggregates

Entamoeba histolytica trophozoites have the capacity to adhere erythrocytes or other cells to their surface, a phenomenon also known as rosette formation (Ravdin and Guerrant, 1981). In the control G3-trophozoites, about 70% of cells were found to bind aggregates of erythrocytes (adherence of more than four erythrocytes in a cluster) after 5 min of incubation and 25% had at least one to four erythrocytes bound separately at the surface. In only 5% of G3-trophozoites no erythrocytes were seen at the amoeba surface (Fig. 4, Table 3). Similar results were obtained with cells in which *ehcp-a1* or *ehcp-a1* and *ehcp-a7* were silenced. In contrast, aggregation of erythrocytes was substantially decreased by 70% and 98% in amoebae silenced for *ehcp-a5* or for all major *ehcp* genes respectively. The majority of these transfectants showed a binding of single erythrocytes only (Fig. 4, Table 3).

Discussion

In an attempt to assign specific CPs of *E. histolytica* trophozoites to biological functions we have generated a number of amoeba lines silenced for the highly expressed *ehcp* genes *ehcp-a1*, *ehcp-a2* and *ehcp-a5* respectively. Using a derivative of the silencing vector psAP1 we were able to reproduce silencing of *ehcp-a5* as previously reported (Bracha *et al.*, 2006). However, careful expression analysis indicated that the *ehcp5*-silenced cells were also silenced for *ehcp-a4*, *ehcp-a6* as well as *ehcp-a8*, which are usually expressed at low levels. Likewise, silencing of *ehcp2* resulted in silencing of all four major *ehcp* genes as well as the three *ehcp* genes mentioned above, namely *ehcp-a4*, *ehcp-a6* and *ehcp-a8* respectively. Moreover, silencing of *ehcp-a1* revealed cell lines silenced for *ehcp-a1* and *ehcp-a7*. The reason for this across-the-board silencing is currently not known, but it is conspicuous that especially those *ehcp* genes are co-silenced that share extensive sequence homologies with the primary target gene. Although this unwanted

Table 3. Binding of erythrocytes to *cp*-silenced transfectants.

	Amoebae (%)		
Transfectants	A (> 4)[a]	S (≤ 4)[b]	⦸[c]
pNSi (control)	68 ± 12	26 ± 8	6 ± 5
pNSicp-a5	30 ± 8**	60 ± 12**	10 ± 10
pNSicp-a1:C1	60 ± 12	40 ± 12	0 ± 0*
pNSicp-a1:C2	55 ± 7	27 ± 8	18 ± 7*
pNSicp-a2	2 ± 2***	54 ± 10**	44 ± 11***

a. The percentage of amoebae binding aggregates of erythrocytes (more than four erythrocytes in a cluster).
b. The percentage of amoebae binding one to four erythrocytes separately.
c. The percentage of amoebae that do not bind erythrocytes.
***$P < 0.0006$, **$P < 0.005$, *$P < 0.04$.

silencing interferes with a straightforward interpretation, the copious set of mutants generated allows to decipher the physiological significance of specific combinations of selected EhCPs. Moreover, this set of mutants enabled the assignment of particular enzymes to specific CP activity bands in substrate gels. It is puzzling that EhCPs belonging to the family EhCP-A, which all have similar molecular masses, differ in their migration behaviour in substrate gel electrophoresis, yielding major activities at 48, 36, 26 and 17 kDa respectively (Hellberg *et al*., 2000; Tillack *et al*., 2006; 2007). The different migration behaviour of the CPs is not yet understood. Nevertheless, using the various silenced mutants we confirmed earlier findings that the 48 kDa activity band is attributed to EhCP-A1 and the 36 kDa activity band to EhCP-A2 (Hellberg *et al*., 2000; 2001). However, in contrast to previous suggestions EhCP-A5 is attributed to an activity band of about 17 kDa and EhCP-A7, which has not been considered before, is attributed to an activity band at 26 kDa (Hellberg *et al*., 2000; Tillack *et al*., 2006; 2007). This result together with the high expression level of *ehcp-a7* measured using real-time PCR indicates that EhCP-A7 is the forth major CP beside EhCP-A1, -A2 and -A5. The reason for the mis-assignment of EhCP-A5 in the previous studies is most likely due to the low amount of EhCP-A5 activity in *E. histolytica* lysates as well as differences in resolution of previous gels. It is known that EhCP-A5 is localized not only in cytoplasmic granules but also on the surface of *E. histolytica* (Jacobs *et al*., 1998). So far it is unknown which part of the total EhCP-A5 is surface associated. In this study, we used only the soluble EhCP-A5 fraction for analyses.

Nevertheless, our result is in contrast to the report by Bracha and colleagues, in which a 30% reduction of CP activity was observed in *ehcp-a5*-silenced amoebae (Bracha *et al*., 2006). This discrepancy may be attributed to the ignorance of possible co-silencing of other major *ehcp* genes in this earlier study.

In several studies a correlation between CP activity and cytopathic activity was postulated (for review see Que and Reed, 2000). It was shown that amoebic lysates as well a purified EhCPs destroy cell monolayers effectively and that this disruption can be inhibited by the CP inhibitor E-64 (Hellberg *et al*., 2001). Nevertheless, it was never shown directly that the cytopathic effect observed using live *E. histolytica* trophozoites is indeed due to EhCPs. The G3-trophozoites used in this study and which are silenced for amoebapore A have a reduced capacity to disrupt cell monolayers (Bracha *et al*., 2003). However, additional silencing of the major *ehcp* genes in these cells did not influence cell monolayer disruption, suggesting that the various peptidases are dispensable for cytopathic activity. This would be consistent with the results from other studies. Ankri *et al*. (1998) have shown that amoebae transfected with *ehcp* antisense constructs and which have only 10% of the total EhCP activity left fully retain their cytopathic activity. Likewise, cultivation of trophozoites in the presence of the CP inhibitor E-64 had only minor effects on cytopathic activity (20% inhibition) (Hellberg *et al*., 2001). Moreover, overexpression of *ehcp-a1* and/or *ehcp-a2* did not increase monolayer disruption (Hellberg *et al*., 2001; Tillack *et al*., 2006). Only massive overexpression of three major CPs (*ehcp-a1*, *-a2*, *-a5*) by about sixfold resulted in significant increase in cytopathic activity (Tillack *et al*., 2006). Thus, it is likely that EhCPs are not the major factors required for disruption of cell monolayers *in vitro*.

The *ehcp*-silenced amoebae remained also unaltered concerning rate of erythrophagocytosis, which is in contrast to other studies. Ankri and colleagues have shown a significant reduction in the rate of phagocytosis in amoebae transfected with *ehcp* antisense constructs (Ankri *et al*., 1998). The same holds true for L6, a peptidase-deficient *E. histolytica* (Orozco *et al*., 1983). This discrepancy may be due to mechanisms inhibiting phagocytosis in the antisense transfectants as well as in the L6 amoeba cell line which are independent of *cp* expression. At least for the L6 cell line it was recently described that the expression of a gene coding for a calcium-binding protein (EhCABP1) is significantly decreased (Hirata *et al*., 2007). This molecule colocalizes with polymerized F-actin and has a central role in controlling the remodelling of membranes during phagocytosis (Sahoo *et al*., 2004).

It was already shown that erythrocyte digestion can be inhibited using a CP inhibitor (Mora-Galindo *et al*., 2004). This was in good agreement with the result using the *cp*-silenced transfectants, which show a high correlation between the amount of CPs and the digestion of erythrocytes.

Also, in contrast to the work of Ankri and colleagues we observed an influence on haemolytic activity for amoebae silenced for all peptidases as well as for *ehcp-a5* and the low-expressed *ehcp-a4*, *-a6* and *-a8* (Ankri *et al*., 1998). It can be speculated that this activity may be attributed to the surface localization of EhCP-A5 (Jacobs *et al*., 1998).

If amoebae adhere to cells such as CHO cells and erythrocytes, rosette formation of the adherent cells is observed. This contact is predominantly mediated by an *N*-acetylgalactosamine-inhibitable lectin located on the surface of the amoebae. Therefore, using *N*-acetyl-D-galactosamine the adherence of amoebae to CHO cells and human red blood cells can be inhibited (Ravdin and Guerrant, 1981; Lotter and Tannich, 1997). Surprisingly, a reduced rosette formation was observed in trophozoites that are silenced in *ehcp-a5* expression and the rosette formation was completely inhibited in pNSicp-a2-transfectants that have no more CP activity. The connection between CPs and erythrocyte aggregation is

ambiguous. The strong reduction of aggregate formation in amoebae where among others EhCP-A5 is missing (pNSicp-a5- and pNSicp-a2-transfectants) may indicate that this surface-associated peptidase is required for processing surface molecules of the amoebae or of target cells which may be necessary to permit aggregation.

Taken together, the results presented here using *E. histolytica* trophozoites silenced for the major *ehcps* genes strongly suggest that this class of enzymes is required for rosette formation, haemolysis and digestion of erythrocytes but dispensable for phagocytosis and cytopathogenicity.

Experimental procedures

Cultivation of cells

The *E. histolytica* isolate HM-1:IMSS:2411, which is needed for transcriptional silencing, was provided by David Mirelman (Department of Biological Chemistry, Weizman Institute of Science, Israel). Trophozoites of the *E. histolytica* isolate HM-1:IMSS:2411 were cultured axenically in TYI-S-33 medium in plastic tissue culture flasks (Diamond *et al*., 1978). For individual experiments 1×10^6 trophozoites were cultivated for 24 h in 75 ml culture flasks. Then, after chilling on ice for 5 min, the trophozoites were harvested by sedimentation at 430 *g* at 4°C for 5 min. The resulting cell pellet was washed twice either in phosphate-buffered saline (PBS) (6.7 mM $NaHPO_4$, 3.3 mM NaH_2PO_4, 140 mM NaCl, pH 7.2) or in TYI-S-33 medium without serum. For preparation of soluble amoeba extracts, cells were lysed by four freeze–thaw cycles in CO_2/ethanol and sedimented by centrifugation (15 000 *g* at 4°C for 15 min).

Silencing constructs

Plasmid psAP1Δ2600 was generated by removing a 2618 bp XbaI fragment from the silencing plasmid psAP1 (Bracha *et al*., 2003). psAP1Δ2600, which lacks the EY tRNA array (glutamic acid$^{(TTC)}$ and tyrosine$^{(GTA)}$) as well as a partial Tet repressor sequence and part of the *E. histolytica* actin gene 3′-sequence that is flanking the neomycin phosphotransferase gene (*neo*), was used as backbone for generating the silencing plasmid pNSi. A PCR-amplified fragment bearing a smaller domain of the EY tRNA array was cloned into TOPO TA vector (Invitrogen), sequenced, and directionally cloned into psAP1Δ2600 digested with XbaI and NotI. In a next step the *ap-a* cassette was removed by digestion with SacII and the vector was religated. Subsequently, a PCR-amplified *ap-a 5′* fragment omitting the SINE sequence (340 bp) was cloned into the NotI site. The genes of interest (*ehcp-a1*, *-a2*, *-a5*) were amplified by PCR, cloned into TOPO TA vector, sequenced, and cloned into pNSi using the restriction sites SacII and SacI (pNSicp-a1, -a2, -a5).

Transfection

Transfections were performed by electroporation as described previously (Hamann *et al*., 1995). To generate amoebae, which are deficient in amoebapore A (Ap-A), trophozoites of the isolate HM-1:IMSS:2411 were transfected with plasmid psAP1 (Bracha *et al*., 2003). Two days after transfection cells were selected with 10 μg ml^{-1} G418 (PAA) for approximately 2 months. Silencing of the *ap-a* gene was confirmed by real-time PCR of reverse transcribed total RNA. Subsequently, cells were cultivated for additional 4 months without the addition of G418 until the episomal plasmid was lost. Loss of the plasmid and maintenance of *ehap-a* silencing were confirmed by real-time PCR and testing for sensitivity against G418. The transgenic amoebae silenced for *ehap-a* (G3-trophozoites) were then transfected with the respective plasmids (pNSicp-a1, pNSicp-a2, pNSicp-a5) leading to silencing of the different *cps*. Directly after transfection the cells were cloned and cultivated in the presence of 10 μg ml^{-1} G418 for approximately 4 weeks. Successful silencing of at least three clones was checked by real-time PCR. The transfectants were further cultivated without the addition of G418. The maintenance of *cp* silencing was confirmed by real-time PCR.

Real-time PCR

A total of 1×10^6 *E. histolytica* trophozoites were cultivated in 75 ml culture flasks for 24 h. The cells were harvested via chilling on ice for 5 min and sedimented at 200 *g* for 5 min at 4°C. The cell pellet was washed twice with PBS. For isolation of total RNA, trophozoites were treated with TRIZOL reagent (Invitrogen) following the manufacturer's instructions. Extracted RNA was further purified using the RNeasy mini kit (Qiagen) omitting β-mercaptoethanol and DNA was digested with DNase (Qiagen). cDNA synthesis was accomplished with SuperScriptIII Reverse Transcriptase (Invitrogen). In a final volume of 20 μl, 1 μg of RNase-free and DNase-treated total RNA was mixed with 5× First-Strand buffer, 500 μM dNTPs, 500 nM OdT-T71 (5′-GAG AGA GGA TCC AAG TAC TAA TAC GAC TCA CTA TAG GGA GAT_{24}), 2 mM DTT, 40 U RnaseOut (Invitrogen) and SuperScriptIII (200 U μl^{-1}). cDNA was synthesized for 1 h at 42°C. For real-time PCR experiments sense and antisense primer were designed amplifying approximately 100–120 bp of the accordant gene (Primer list: Table S1 in *Supporting information*). Quantitative amplification was performed in a Rotor-Gene PCR apparatus (Corbett) using RealMasterMix SYBR Rex kit (5 PRIME). One microlitre of cDNA was mixed with 2.5× RealMasterMix/20× SYBR and 5 pmol μl^{-1} appropriate sense and antisense primers to a final volume of 20 μl. Amplification conditions were as follows: 35 cycles at 95°C for 15 s, 58°C for 20 s and 68°C for 20 s and an adjacent melting step (67– 95°C). Two biological replicates were analysed in duplicates. Relative differences in gene expression between G3-trophozoites and respective transfectants were calculated using the $2^{-\Delta\Delta CT}$ method provided by the Rotor-Gene software (Livak and Schmittgen, 2001). G3-trophozoites represented the calibrator cell line and actin was chosen as the housekeeping gene for normalization. Efficiency ≥ 0.95 was empirically approved for selected primer and cDNA. Threshold for silencing expressed genes was set on 0.2.

Microarray

For microarray experiments a 60-base oligonucleotide array, recently described containing 80 putative peptidase genes,

was used (Tillack *et al.*, 2007). RNA isolation, microarray hybridization, sample labelling and visualization were performed as described (Tillack *et al.*, 2007). In summary, total amoeba RNA was isolated using TRIZOL reagent (Invitrogen) according to standard protocols. For microarray analysis 16 μg of total RNA was used. The reverse transcription of RNA into cDNA was performed according to the Superscript Indirect cDNA Labeling System (Invitrogen), followed by indirect labelling with Cy3- and Cy5-monoreactive dyes (Amersham). Two biological replicates including dye swap experiments were performed. Pre-hybridization and hybridization were performed using standard protocols. Each array was scanned at 550 nm (Cy3) and at 650 nm (Cy5) at a resolution of 5 μm. Calculation and output of the data were performed using ScanArray software, version 3.0 (PerkinElmer). For calculation, the mean signal intensity (pixel) minus local background (pixel) of each spot was used. Two methods for normalizing the data were applied: (i) normalization among the totality of genes and (ii) normalization among housekeeping genes.

Enzymatic assay

Proteolytic activity was measured using the synthetic peptide Z-Arg-Arg-pNA · 2HCl [(Z = Cbz = Benzyloxycarbonyl; pNA = *p*-nitroanilide) Bachem] as substrate (Leippe *et al.*, 1995). One unit of enzymatic activity is defined as the amount that catalyses the reduction of 1 μmol min^{-1} of *p*-nitroaniline. The activity was measured four times in duplicate.

Substrate gel electrophoresis

The substrate gel electrophoresis was performed as described previously (Hellberg *et al.*, 2000). In summary, 2 μg of amoebic extract was incubated with Laemmli buffer containing 20 mM DTT for 5 min at 37°C. For the substrate gel, a 12% SDS-polyacrylamide gel was co-polymerized with 0.1% gelatine. After separation of the proteins and incubation in buffer A (2.5% Triton X-100; 1 h) and buffer B (100 mM sodium acetate, pH 4.5, 1% Triton X-100, 20 mM DTT; 3 h) at 37°C the gel was stained with Coomassie blue.

Quantification of cytopathic activity

Interaction of trophozoites and CHO cells was determined according to Bracha and Mirelman (1984) with some modifications. CHO cells (1×10^5) were grown for 48 h in 24-well plates in Ham's F12 medium supplemented with 10% inactivated fetal calf serum and 1% penicillin/streptomycin (100×, all substances from PAA). After washing the CHO cells with pre-warmed (37°C) Ham's F12, 500 μl of medium was added to the cells. The trophozoites (1×10^5) were washed twice with serum-free TYI-S-33 medium, re-suspended in 300 μl of serum-free TYI-S-33 and added to the CHO cells. The cells were incubated for 90 min at 37°C. The amount of CHO cells that remained attached was proportional to 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 twice in triplicates.

Haemolytic activity

Human erythrocytes (group O Rh^+) and *Entamoeba* trophozoites were washed three times with PBS. The assay was performed by mixing trophozoites and erythrocytes in a ratio of 1:2000 (2.5×10^5 amoebae with 5×10^8 erythrocytes per ml of PBS) followed by incubation for 1 h at 37°C. After incubation the cells were sedimented for 1 min at 2000 *g*. The haemoglobin that was released in the supernatant was read at 570 nm in a spectrophotometer. As negative controls erythrocytes and trophozoites were incubated separately. The experiment was performed three times in triplicates.

Phagocytosis and digestion of erythrocytes

A total of 5×10^5 trophozoites per well (1 ml) were cultivated for 24 h in a 24-well plate at 36°C under anaerobic conditions. After removing the medium 1 ml of TYI-S33 medium containing 1×10^7 erythrocytes was added to the wells and incubated for additional 1 h under anaerobic conditions using Anarocult (Merck). Afterwards the medium was completely removed and the erythrocytes were lysed by the addition of 1 ml of water (37°C). For each experiment two identical plates have to be prepared. In one plate the remaining amoebae were re-suspended in 100 μl of incomplete TYI-S33 medium (without serum) and the number of phagocytosed erythrocytes was determined by light microscopy. In the parallel plate 1 ml of TYI-S33 medium was added and the amoebae were incubated for additional 2 h under anaerobic conditions at 36°C. Afterwards the medium was removed and the amoebae were re-suspended in 100 μl of incomplete TYI-S33 medium and the number of phagocytosed erythrocytes was determined by light microscopy. The experiment was performed five times in duplicates.

Erythrocyte aggregation

A total of 1×10^6 amoeba trophozoites were cultivated in TYI-S33 for 24 h, before they were harvested via chilling on ice for 5–10 min in 1 ml of medium. Ten microlitres of the cell suspension was mixed with 1 μl of human erythrocytes (containing approximately 5×10^6 cells) and applied onto a slide. Binding of erythrocytes to amoebae was detected directly by light microscopy. The experiment was performed four times (every time approximately 50 amoebae per silencing cell line were counted). The percentage of amoebae binding aggregates of erythrocytes (more than four erythrocytes in a cluster), one to four single erythrocytes and no erythrocytes was determined.

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References

Ankri, S., Stolarsky, T., and Mirelman, D. (1998) Antisense inhibition of expression of cysteine proteinases does not affect *Entamoeba histolytica* cytopathic or haemolytic activity but inhibits phagocytosis. *Mol Microbiol* **28:** 777–785.

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.

Bracha, R., and Mirelman, D. (1984) Virulence of *Entamoeba histolytica* trophozoites. Effects of bacteria, microaerobic conditions, and metronidazole. *J Exp Med* **160:** 353–368.

Bracha, R., Nuchamowitz, Y., and Mirelman, D. (2003) Transcriptional silencing of an amoebapore gene in *Entamoeba histolytica*: molecular analysis and effect on pathogenicity. *Eukaryot Cell* **2:** 295–305.

Bracha, R., Nuchamowitz, Y., Anbar, M., and Mirelman, D. (2006) Transcriptional silencing of multiple genes in trophozoites of *Entamoeba histolytica*. *PLoS Pathog* **2:** e48.

Bruchhaus, I., Loftus, B.J., Hall, N., and Tannich, E. (2003) The intestinal protozoan parasite *Entamoeba histolytica* contains 20 cysteine protease genes, of which only a small subset is expressed during *in vitro* cultivation. *Eukaryot Cell* **2:** 501–509.

Clark, C.G., Alsmark, U.C., Tazreiter, M., Saito-Nakano, Y., Ali, V., Marion, S., *et al.* (2007) Structure and content of the *Entamoeba histolytica* genome. *Adv Parasitol* **65:** 51–190.

Diamond, L.S., Harlow, D.R., and Cunnick, C.C. (1978) A new medium for axenic cultivation of *Entamoeba histolytica* and other *Entamoeba*. *Trans R Soc Trop Med Hyg* **72:** 431–432.

Gadasi, H., and Kessler, E. (1983) Correlation of virulence and collagenolytic activity in *Entamoeba histolytica*. *Infect Immun* **39:** 528–531.

Hamann, L., Nickel, R., and Tannich, E. (1995) Transfection and continuous expression of heterologous genes in the protozoan parasite *Entamoeba histolytica*. *Proc Natl Acad Sci USA* **92:** 8975–8979.

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.

Hirata, K.K., Que, X., Melendez-Lopez, S.G., Debnath, A., Myers, S., Herdman, D.S., *et al.* (2007) A phagocytosis mutant of *Entamoeba histolytica* is less virulent due to deficient proteinase expression and release. *Exp Parasitol* **115:** 192–199.

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.

Keene, W.E., Hidalgo, M.E., Orozco, E., and Mckerrow, J.H. (1990) *Entamoeba histolytica*: correlation of the cytopathic effect of virulent trophozoites with secretion of a cysteine proteinase. *Exp Parasitol* **71:** 199–206.

Leippe, M., Sievertsen, H.J., Tannich, E., and Horstmann, R.D. (1995) Spontaneous release of cysteine proteinases but not of pore-forming peptides by viable *Entamoeba histolytica*. *Parasitology* **111:** 569–574.

Li, E., Yang, W.G., Zhang, T., and Stanley, S.L., Jr (1995) Interaction of laminin with *Entamoeba histolytica* cysteine proteinases and its effect on amebic pathogenesis. *Infect Immun* **63:** 4150–4153.

Livak, K.J., and Schmittgen, T.D. (2001) Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) method. *Methods* **25:** 402–408.

Lotter, H., and Tannich, E. (1997) The galactose-inhibitable surface lectin of *Entamoeba histolytica*, a possible candidate for a subunit vaccine to prevent amoebiasis. *Behring Inst Mitt* **99:** 112–116.

Luaces, A.L., and Barrett, A.J. (1988) Affinity purification and biochemical characterization of histolysin, the major cysteine proteinase of *Entamoeba histolytica*. *Biochem J* **250:** 903–909.

Lushbaugh, W.B., Hofbauer, A.F., and Pittman, F.E. (1985) *Entamoeba histolytica*: purification of cathepsin B. *Exp Parasitol* **59:** 328–336.

Mirelman, D., Anbar, M., and Bracha, R. (2008) Epigenetic transcriptional gene silencing in *Entamoeba histolytica*. *IUBMB Life* **60:** 598–604.

Mora-Galindo, J., Anaya-Velazquez, F., Ramirez-Romo, S., and Gonzalez-Robles, A. (2004) *Entamoeba histolytica*: correlation of assessment methods to measure erythrocyte digestion, and effect of cysteine proteinases inhibitors in HM-1:IMSS and HK-9:NIH strains. *Exp Parasitol* **108:** 89–100.

Orozco, E., Guarneros, G., Martinez-Palomo, A., and Sanchez, T. (1983) *Entamoeba histolytica*. Phagocytosis as a virulence factor. *J Exp Med* **158:** 1511–1521.

Que, X., and Reed, S.L. (2000) Cysteine proteinases and the pathogenesis of amebiasis. *Clin Microbiol Rev* **13:** 196–206.

Ravdin, J.I., and Guerrant, R.L. (1981) Role of adherence in cytopathogenic mechanisms of *Entamoeba histolytica*. Study with mammalian tissue culture cells and human erythrocytes. *J Clin Invest* **68:** 1305–1313.

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.

Sahoo, N., Labruyere, E., Bhattacharya, S., Sen, P., Guillen, N., and Bhattacharya, A. (2004) Calcium binding protein 1 of the protozoan parasite *Entamoeba histolytica* interacts with actin and is involved in cytoskeleton dynamics. *J Cell Sci* **117:** 3625–3634.

Schulte, W., and Scholze, H. (1989) Action of the major protease from *Entamoeba histolytica* on proteins of the extracellular matrix. *J Protozool* **36:** 538–543.

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.

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.

Tillack, M., Biller, L., Irmer, H., Freitas, M., Gomes, M.A., Tannich, E., and Bruchhaus, I. (2007) The *Entamoeba histolytica* genome: primary structure and expression of proteolytic enzymes. *BMC Genomics* **8:** 170.

WHO (1997) WHO/PAHO/UNESCO report. A consultation with experts on amoebiasis. Mexico City, Mexico 28–29 January, 1997. *Epidemiol Bull* **18:** 13–14.

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