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Oxidative stress resistance genes contribute to the pathogenic potential of the anaerobic protozoan parasite, *Entamoeba histolytica*

Elena Rastew^a, João B. Vicente^{a,c}, and Upinder Singh^{a,b,*}

^aDepartment of Internal Medicine, Stanford University School of Medicine, Stanford, CA, USA

^bDepartment of Microbiology and Immunology, Stanford University School of Medicine, Stanford, CA, USA

Abstract

The protozoan parasite, *Entamoeba histolytica*, invades the host colon causing significant tissue destruction and inflammation. Upon host infection, the parasite is confronted with reactive oxygen and nitrogen species (ROS/RNS) that cause large-scale changes in gene expression profiles, which likely support the parasite's adaptation to the host environment. We have previously identified oxidative and nitrosative stress responsive genes using whole-genome expression profiling. Functional studies on two such genes are now reported and demonstrate that they have roles in parasite virulence. EHI_056680 encodes a small hypothetical protein named *E. histolytica* stress-induced adhesion factor (EhSIAF); EHI_188210 encodes a putative phospholipid transporting P-type ATPase/flippase (EhPTPA). Over-expression of each protein in *E. histolytica* trophozoites enhanced parasite survival in response to oxidative stress. Exposure to oxidative and nitrosative stress did not affect the localization of EhSIAF or EhPTPA but markedly increased EhPTPA protein levels. Interestingly, over-expression of each gene resulted in parasites with increased adherence to healthy mammalian cells, but increased adherence to apoptotic cells was noted only in EhSIAF over-expressing parasites. However, despite having increased adherence to both healthy and apoptotic host cells, EhSIAF-over-expressing parasites were reduced in their ability to destroy mammalian cell monolayers, raising the intriguing possibility that EhSIAF over-expression caused signaling defects or resulted in a dominant negative phenotype. Over-expression of EhSIAF and EhPTPA also resulted in decreased motility in a transwell motility assay. Thus, we have confirmed that two genes that are upregulated by ROS confer increased resistance to oxidative stress and have identified an unexpected role of EhSIAF and EhPTPA in host cell adherence and a role of EhSIAF in parasite virulence. Our data imply that stress response genes may play multi-factorial roles in amoebic pathogenesis.

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*Corresponding author. Upinder Singh, S-143 Grant Building, 300 Pasteur Drive, Stanford University, Stanford, CA, USA 94305. Tel.: +1-650-723-4045; fax: +1-650-724-3892. usingh@stanford.edu.

^cPresent address - Metabolism and Genetics Group, Institute for Medicines and Pharmaceutical Sciences (iMed.UL), Faculdade de Farmácia da Universidade de Lisboa, Lisbon, Portugal

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Keywords

Entamoeba histolytica; Reactive oxygen species; Reactive nitrogen species; Virulence; Cytotoxicity

1. Introduction

Entamoeba histolytica is the etiological agent of amoebiasis and a leading cause of death from parasitic diseases (Stanley, 2003; Salles et al., 2007). The *E. histolytica* life cycle alternates between an inert, infectious cyst form and a motile, invasive trophozoite form. Transmitted by a fecal-oral route, ingested cysts travel to the small intestine where they excyst to invasive trophozoites. *Entamoeba histolytica* trophozoites can penetrate mucosal and submucosal tissues of the large intestine by adhering to and disrupting the intestinal epithelial barrier, with symptoms ranging from severe bloody diarrhea to extraintestinal tissue invasion (Stanley, 2003).

Upon host invasion, *E. histolytica* must cope with oxidative and nitrosative stress from various sources including varying oxygen tensions within the intestinal lumen and reactive oxygen and nitrogen species (ROS and RNS) produced by the immune system in response to pathogen invasion and tissue destruction. A correlation between oxidative and nitrosative stress resistance and amoebic pathogenesis has been postulated for *E. histolytica* on the basis that (i) virulent strains and species of *Entamoeba* have higher transcript levels of genes involved in oxidative stress resistance (MacFarlane and Singh, 2006), (ii) virulent strains display a higher resistance to oxygen exposure (Ramos-Martinez et al., 2009) and oxidative and nitrosative stress (Biller et al., 2009, 2010), (iii) a large set of genes are modulated in response to oxidative and nitrosative stress (Akbar et al., 2004; Vicente et al., 2009; Santi-Rocca et al., 2012), and (iv) a proteomic analysis of two *E. histolytica* strains with different pathogenic potential revealed that the more virulent strain has higher levels of ROS-scavenging proteins (Biller et al., 2009). Furthermore, transcriptional studies identified significantly higher numbers of ROS- and RNS-responsive genes in the pathogenic *E. histolytica* HM-1:IMSS strain compared with the non-pathogenic *E. histolytica* Rahman strain, strongly indicating a correlation between stress response and pathogenic potential (Vicente et al., 2009). The most dramatic and significant changes were related to signaling/regulation pathways and repair of misfolded proteins, nucleic acids and lipids (Vicente et al., 2009). The list of stress-modulated genes also includes several candidates related to virulence-related cellular processes such as adherence to host cells and ability to destroy and phagocytose both human and bacterial cells. However, the majority of the ROS and RNS responsive genes (especially the genes with the highest induction levels) code for hypothetical proteins of unknown function, which underlines the fact that the molecular basis of amoebic stress response remains largely unknown.

Herein, we report the functional characterization of two genes (EHI_056680 and EHI_188210) that were induced by oxidative and nitrosative stress (Vicente et al., 2009). EHI_056680 encodes for a hypothetical protein with no significant homologies or well-described domains. Based on its functional characterization we have named it *E. histolytica* stress-induced adhesion factor (EhSIAF). EHI_188210 encodes a phospholipid transporting P-type ATPase/flippase (EhPTPA). The functions of both genes were studied by generating stable transfectant *E. histolytica* cell lines over-expressing Myc-tagged versions of each protein for phenotypic analyses. Whereas Myc-EhPTPA was located on internal membranes, Myc-EhSIAF was predominantly cytoplasmic, although also accumulating near membranes. Interestingly, Myc-EhPTPA protein levels increased dramatically upon exposure to oxidative and nitrosative stress. Over-expression of EhSIAF and EhPTPA enhanced parasite

survival when exposed to exogenous hydrogen peroxide. Both proteins afforded increased adherence to healthy mammalian cells, whereas only EhSIAF-over-expressing parasites displayed an increased adherence to apoptotic cells. However, despite having increased adherence to healthy or apoptotic cells, EhSIAF over-expressing amoebae were significantly impaired in their ability to destroy mammalian cell monolayers. Our findings imply multifactorial roles of these genes in amoebic pathogenesis. Overall, our data demonstrate the sophisticated relationship between the parasite's response to oxidative stress and its virulence potential.

2. Materials and methods

2.1. Sequence analysis

The primary sequences for EhSIAF (EHI_056680) and EhPTPA (EHI_188210) were retrieved from the Pathema website (<http://pathema.jcvi.org/cgi-bin/Entamoeba/PathemaHomePage.cgi>). EhPTPA sequence alignment was performed with ClustalX for Windows (Thompson et al., 1997), after selected homologue sequences were retrieved from NCBI BLAST (<http://www.ncbi.nlm.nih.gov/BLAST>). No homologues for EhSIAF were retrieved from a search using the same server. Secondary structure and transmembrane topology predictions were obtained from the PSIPRED and MEMSAT-SVM servers (Jones, 1999; Nugent and Jones, 2009). Prediction of EhPTPA N-glycosylation sites was retrieved from the NetNGlyc 1.0 server (Blom et al., 2004) as well as from ScanProsite server (Marshall, 1972).

2.2. Plasmid construction to obtain transgenic amoebic lines

The full-length coding regions of EhSIAF and EhPTPA were amplified by PCR from *E. histolytica* HM-1:IMSS genomic DNA using the primers listed in Supplementary Table S1, and cloned into a Topo TA pCR®2.1 vector (Invitrogen, USA). The PCR fragment for EhSIAF was digested with *Sma*I and *Xho*I restriction enzymes, whereas EhPTPA was digested with *Nhe*I (New England Bio Labs Inc., USA). After gel purification EhSIAF was sub cloned into pKT-3M at the *Sma*I and *Xho*I restriction sites resulting in an N-terminal triple Myc tag fusion, while EhPTPA was subcloned into pEhEx-3M at the *Nhe*I site yielding a C-terminal triple Myc tag fusion. Both vectors have routinely been used by the *E. histolytica* community and contain the cysteine synthase promoter and regulatory regions driving expression of the cloned genes (Saito-Nakano et al., 2004). As suggested by our transcriptomics analysis (Vicente et al., 2009), the cysteine synthase promoter is not stress responsive. Correct gene insertion was confirmed by sequencing.

2.3. Generation and maintenance of stable transfectants

Entamoeba histolytica HM-1:IMSS trophozoites were transfected as previously described (Saito-Nakano et al., 2004; Baxt et al., 2010). Briefly, mid-log parasites were seeded into 25 mm Petri dishes, sealed with parafilm to minimize the oxygen exposure and allowed to grow for 24 h. Transfection mixture containing 20 µg of plasmid DNA and 20 µl of SuperFect (Qiagen, USA) in a total volume of 200 µl of M199 medium (Gibco, USA) was incubated for 10 min at room temperature (RT). Plated amoebae were washed with M199 medium, after which 2 ml of M199 supplemented with 15% heat-inactivated bovine serum (Sigma-Aldrich, USA) was added to the plate. The SuperFect-DNA mixture was pipetted onto the plated amoebae, the dishes sealed with parafilm and incubated at 37°C for 4 h, iced for 5 min to release parasites from the dish and transferred to a 15 ml glass tube containing fresh TYI (Trypticase, yeast extract, iron-serum) medium (Diamond et al., 1978). Parasites were allowed to grow for 48 h before adding 2 µg/ml of neomycin (Cellgro, USA). The neomycin concentration was increased stepwise until stably transfected parasite lines at 24 µg/ml were obtained.

2.4. Western blot analysis

Lysates were prepared using log phase trophozoites. Trophozoites were iced for 5 min, pelleted at 1,000 *g* for 5 min at 4°C followed by a single wash in ice cold PBS. EhSIAF over-expressing parasites were resuspended in lysis buffer (50 mM HEPES-KOH pH7.5, 50 mM KCl, 5 mM MgCl₂, 0.5% NP-40) containing protease inhibitors (2 mM DTT, 50 μM E-64 (Sigma-Aldrich), 0.4 μg/ml of Leupeptin (Sigma-Aldrich), 2 x HALT inhibitor cocktail (#78410 Thermo Scientific, USA)). EhPTPA over-expressing parasites were resuspended in NETN lysis buffer (100 mM NaCl, 20 mM Tris pH 8.0, 1 mM EDTA, 0.2% NP-40) containing 50 μM E-64 and 1 x HALT inhibitor cocktail. After an incubation period of 10 min on ice and centrifugation at 14,000 *g* for 10 min, the protein concentration was determined by the Bradford method. EhSIAF lysates were applied to a 15% SDS-PAGE gel and transferred onto a polyvinylidene fluoride (PVDF) membrane (#162-0174 BioRad, USA). EhPTPA lysates were run on a 6% SDS-PAGE gel and transferred to nitrocellulose membrane. The membranes were blocked with 5% milk in PBS-T (PBS containing 0.1% Tween-20). Blots were incubated with antibodies against Myc (1:1,000 dilution; Cell Signaling Technology, USA) and Actin (1:1,000 dilution; MP Biomedicals, USA), followed by incubation with secondary mouse horseradish peroxidase (HRP)-conjugated antibody (1:1,000 dilution; Cell Signaling Technology or Santa Cruz Biotechnology, USA) and developed using enhanced chemiluminescence (ECL) (GE Healthcare, USA). Blots were either scanned on a Kodak Image Station 4000R or imaged on film and developed on Kodak X-OMAT 2000 processor.

2.5. Immunofluorescence assays

Immunofluorescence microscopy assays were performed as previously described (Baxt et al., 2008). Briefly, parasites were seeded into chamber slides (LabTek, USA), allowed to adhere at 37°C for 30 min, rinsed with 1 x PBS, and fixed for 12 min with 4% ultra-pure formaldehyde (PolyScience Inc., USA) diluted in 1 x PBS and 10 mM MgCl₂. Cells were permeabilized with 100% ethanol for 20 min followed by blocking with 3% BSA (in PBS) for 30 min. Cells were stained with primary antibody (anti-Myc mouse monoclonal antibody at 1:250 (Cell Signaling Technology)); anti-Hgl H85 mouse monoclonal antibody at 1:50 (kind gift of WA Petri, Jr., University of Virginia, USA) diluted in 1% BSA (in PBS), incubated at RT for 1 h, and followed by staining with fluorescent secondary antibodies Alexa 594 or Alexa 488 (1:1,000, Invitrogen, USA) in the dark for 30 min at RT. Imaging was performed on a Leica CTR6000 microscope with a BD CARVII confocal unit. Analysis and deconvolution were performed using the LAS-AF software from Leica. Deconvolution was carried out in 10 iterations, with a single slice shown for each sample.

2.6. Sensitivity of parasite strains to oxidative and nitrosative stress and heat shock

Confluent transfected parasites were treated in glass tubes with 1 mM hydrogen peroxide (oxidative stress, EMD Millipore, USA) or 200 μM-500 μM dipropyleneetriamine (DPTA)-NONOate (nitrosative stress, Cayman Chemical, USA) for 60–90 min at 37°C, iced for 5 min and pelleted, and stained with 0.2% Trypan blue dye (Gibco, USA) to assess cell viability. Dead cells stain blue, whereas viable cells exclude the dye. As a non-related marker of general stress, heat shock was applied to the parasites by placing tubes with confluent parasite cultures at 42–45°C for 60–90 min. With all functional studies parasites were intentionally chosen to be under drug selection and expressing a non-amoebic gene. From our observations and experience, parasites harboring a luciferase over-expression vector largely behave similarly to wild type parasites, which encouraged use of this cell line as a control.

2.7. Adherence assays

Adherence assays to healthy mammalian cells were performed using Chinese hamster ovary (CHO) cells as previously described (MacFarlane and Singh, 2007). Briefly, 1×10^4 parasites and 2×10^5 CHO cells were mixed together, centrifuged at 150 *g* for 5 min and incubated on ice for 1 h. After incubation, the supernatant was removed, the remaining 100 μ l of media gently resuspended and a hemocytometer was used to count the parasites. Parasites with three or more attached cells were considered positive for adhesion. Apoptotic CHO cells were generated as previously described using Staurosporine aglycone (Sigma-Aldrich), an established inducer of apoptosis (Baxt et al., 2010). Briefly, CHO cultures were enriched for viable cells by applying Ficoll-Paque PLUS (GE Healthcare, Sweden). Viable cells were collected on top of the Ficoll-Paque layer. Apoptosis was induced by treatment with Staurosporine aglycone dissolved in 1 μ M DMSO (Sigma-Aldrich) for 1 h at 4° C. Control parasites were treated with 1 μ M DMSO.

2.8. Cell monolayer destruction assays

Assays were carried out as previously described (Hellberg et al., 2001). Briefly, 5×10^4 trophozoites were placed on a confluent CHO cell monolayer, centrifuged for 5 min at 50 *g* and incubated for 2 h at 37°C. Cells were fixed with 4% ultra-pure formaldehyde for 10 min and rinsed twice with PBS, followed by staining with 0.1% methylene blue (OmniPur, USA) diluted in 10 mM borate buffer (pH 8.7) and washed three times with the same buffer. The dye was extracted by adding 1 ml of 0.1 M HCl at 37° C for 30 min. In order to measure the extracted dye, samples were diluted 1:10 with PBS and the absorbance at 650 nm was read in a spectrophotometer. The intact monolayer was accounted as 0% of destruction and the syringe-lysed monolayer was set as 100% of destruction and used as a blank.

2.9. Motility assays

For transwell motility assays parasites were grown to confluence in glass tubes. Cells were iced, pelleted and resuspended in complete TYI medium (containing vitamins and serum) at a concentration of 5×10^5 cells/ml. Parasites (1.5×10^5) were added to the top of the transwell insert containing 8 μ m pores (Costar, USA). Complete TYI medium (1 ml) was placed in the bottom of the chamber. The 24 well plate was sealed with parafilm and placed in an anaerobic bag (Becton Dickinson, USA) for 3 h at 37°C. Finally the transwell inserts were removed and the parasites in the bottom chamber iced, transferred to Eppendorf vessels, centrifuged at 1,000 *g* for 5min, resuspended in 20 μ l of TYI medium and quantified with a hemocytometer.

2.10. Red blood cell phagocytosis assays

Phagocytosis of human red blood cells (hRBCs) was assayed as previously published (Mora-Galindo et al., 1997). Briefly, a total of 1×10^8 hRBCs (Stanford Blood Center) were incubated with 1×10^5 trophozoites in 0.2 ml of TYI for 15 min at 37°C. Parasites and hRBCs were pelleted at 1,000 *g* for 2 min and resuspended twice in ice cold distilled water, ensuring lysis of all extracellular hRBCs. Ingested hRBCs were lysed in concentrated formic acid (88%, Sigma-Aldrich) followed by recording absorbance at 405 nm.

3. Results

3.1. ROS and RNS species trigger the upregulation of EhSIAF and EhPTPA in *E. histolytica* trophozoites

Based on microarray analysis we observed that mRNA levels of EhSIAF and EhPTPA were differentially upregulated upon oxidative (EhSIAF, 15-fold and EhPTPA, 2.3-fold) and nitrosative (EhSIAF, 13.5-fold and EhPTPA, 2.6-fold) stress in *E. histolytica* HM-1:IMSS

parasites (Table 1). The high level of upregulation for EhSIAF was largely due to nearly absent basal levels of mRNA in wild type parasites. Interestingly, both EhSIAF and EhPTPA appear to be developmentally regulated, although in opposing ways: EhPTPA transcript is higher in cysts but EhSIAF has higher transcript levels in trophozoites (Ehrenkaufer et al., 2007). The expression of EhSIAF is also upregulated in trophozoites under heat shock (Hackney et al., 2007) and in transfected strains under G418 drug selection, suggesting it may be regulated as a general stress response. Neither gene appears to be modulated significantly during intestinal colonization (Gilchrist et al., 2006).

3.2. Sequence analysis

A search for EhSIAF homologues using NCBI BLAST retrieved a single protein sequence from *Entamoeba dispar* (e-value $5e-71$, 85% identity), with no evidence for homologues in any other genomes. The fact that EhSIAF is unique gives it increased significance in terms of unraveling its function. As predicted with PSIPRED analysis, the EhSIAF sequence has a limited number of secondary structure elements and most of its sequence consists of unstructured coils (Supplementary Fig. S2A). EhPTPA homologues, generally annotated as phospholipid-transporting ATPases, were identified in most organisms ranging from higher eukaryotes to bacteria. A sequence alignment employing selected homologues from different sources (Supplementary Fig. S1) reveals several sequence motifs with significant similarity. Analysis performed by PSIPRED and MEMSAT-SMV servers resulted in identification of eight or nine transmembrane helices within the EhPTPA sequence, depending on the prediction software (Supplementary Fig. S2B). A number of putative N-glycosylation sites were predicted based on the presence of the N-X-S/T consensus sequence as a general recognition element. P-type ATPases are often involved in the maintenance of phospholipid asymmetry and cell polarity and might play a protective role during extreme environmental stress conditions (Chan et al., 2010).

3.3. Over-expression of EhSIAF and EhPTPA in *E. histolytica* trophozoites

In order to examine biologically relevant phenotypes associated with EhSIAF and EhPTPA, we cloned each gene into a vector containing the constitutively active *E. histolytica* cysteine synthase promoter, ensuring expression of a Myc-tagged version of each protein (Saito-Nakano et al., 2004). Whereas EhSIAF bears an N-terminal Myc tag, EhPTPA has a C-terminal Myc tag, since the EhPTPA primary sequence contains a predicted signal peptide. Over-expression of Myc-tagged EhSIAF resulted in positive signal by western blot analysis at the predicted size of 16 kDa, and two extra bands corresponding to potential dimer (32 kDa) and trimer (48 kDa) forms (Fig. 1A, B). The over-expression of the EhSIAF protein did not change significantly with exposure to oxidative or nitrosative stress (Fig. 1A, B). The localization of EhSIAF protein appears to be predominantly cytoplasmic although considerable signal intensity was observed close to cellular membranes (Fig. 1C). Due to the lack of the signal co-localization with Gal/GalNAc lectin on non-permeabilized parasites, it appears that the EhSIAF protein was located on the inner leaflet of the plasma membrane. Exposure to oxidative or nitrosative stress did not affect the localization of EhSIAF (Supplementary Fig. S3).

Western blot analysis of amoebic lysates over-expressing Myc-tagged EhPTPA gave a positive signal only upon parasite exposure to oxidative or nitrosative stress, with a smeary band at the predicted size of a monomer (120 kDa) and another band centered around 240 kDa, corresponding to the predicted size of a dimer (Fig. 2A). One potential explanation is that possible post-translational modifications, resulting from ROS or RNS exposure, had an impact on EhPTPA conformation or translation and detection by anti-Myc antibody, e.g. the exposure of the C-terminal Myc-epitope may occur only upon stress exposure or altered glycosylation. The smeary western blot signal is consistent with the glycosylated status of

EhPTPA, which is in accordance with the NetNGlyc 1.0 Server N-glycosylation sites prediction (Supplementary Fig. S2B). Immunofluorescence microscopy suggests that EhPTPA localizes mostly to internal membranes, with the C-terminal EhPTPA epitope exposed to the cytoplasm (Fig. 2B). Exposure to oxidative and nitrosative stress did not significantly affect the localization of EhPTPA within the parasite (Supplementary Fig. S4). We realize that localization studies using Myc-tagged proteins are approximations. For protein levels by western blot, we aimed to confirm that the transfected cell lines were expressing the target proteins, so that observed phenotypes could be attributed to protein expression. Future studies with antibodies to these proteins will be helpful.

3.4 EhSIAF and EhPTPA-over-expressing strains display increased resistance to hydrogen peroxide

Since both EhSIAF and EhPTPA were induced by ROS and RNS, we assessed whether over-expression of the genes would confer increased resistance to these stresses. It was previously shown that over-expression of *Leishmania* cytoplasmic peroxiredoxin LcPxn1 enhanced survival when exposed to ROS and RNS (Barr and Gedamu, 2003). Davis and colleagues (2006) noted that *E. histolytica* peroxiredoxin is differentially expressed in virulent and non-virulent *E. histolytica* strains and moreover demonstrated that over-expression of peroxiredoxin in the avirulent *E. histolytica* Rahman strain resulted in increased resistance to ROS accompanied by increased virulence in a human intestinal xenograft model. To evaluate whether EhSIAF or EhPTPA is involved in the *E. histolytica* oxidative stress response, transfectants over-expressing each protein were exposed to 1 mM hydrogen peroxide and their viability assessed by trypan blue staining. Viable cells with intact membranes were able to exclude the dye, whereas dead cells were susceptible to the dye and stained blue. *Entamoeba histolytica* transfectants over-expressing EhSIAF or EhPTPA were more resistant to oxidative stress than the control strain (amoebae under drug selection expressing the luciferase protein), with approximately 20% increased survival (P value <0.01) (Fig. 3). This finding is consistent with a protective role of both genes against oxidative stress. This level of protection is similar to what was observed for *E. histolytica* peroxiredoxin, which when over-expressed in the avirulent strain afforded increased resistance to ROS (Davis et al., 2006). Notably, the protective role of both EhSIAF and EhPTPA appears to be specific to oxidative stress, since neither gene afforded any increased survival against heat shock (42–45°C, for 60–90 min) (data not shown). Since the exposure to nitrosative stress (200–500 μ M DPTA NONOate for 60–90 min) resulted in no significant death in control cells, the effect of over-expressing EhSIAF or EhPTPA could not be assessed (data not shown).

3.5 EhSIAF and EhPTPA afford increased adherence to host cells

Under the premise that oxidative and nitrosative stress regulated genes may serve as amoebic virulence determinants, we investigated the roles of EhSIAF and EhPTPA in various virulence-associated phenotypes. Adhesion to host cells is a crucial step in the pathogenesis of *E. histolytica*, since attachment-mediated cytotoxicity is a major cell killing mechanism (Petri et al., 1987; Saffer and Petri, 1991; Huston et al., 2000). To address the effect of EhSIAF or EhPTPA over-expression on the ability of parasites to adhere to healthy CHO cells, we employed a standard CHO cell rosette assay (MacFarlane and Singh, 2007). Notably, EhSIAF and EhPTPA-over-expressing parasites showed significantly increased adherence (P value <0.01) to CHO cells compared with control parasite strains (Fig. 4A). Since adherence was dramatically increased and the surface carbohydrate-binding lectin (Hgl) is strongly implicated in amoebic adherence to the healthy host cells (Petri et al., 1987; Saffer and Petri, 1991), we tested whether Hgl protein levels of EhSIAF and EhPTPA parasites were increased by western blot analysis, but did not observe any significant changes compared with controls (data not shown).

There appear to be specific receptors for amoebic adhesion to healthy and apoptotic cells. The serine-rich *E. histolytica* protein (SREHP) was shown to be the major receptor utilized by parasites during adhesion to apoptotic cells (Teixeira and Huston, 2008), while the phagosome-associated transmembrane kinase (PATMK) appears to play a role in adhesion to both healthy and apoptotic erythrocytes (Boettner et al., 2008). To further explore this enhanced adherence phenotype and to specify whether EhSIAF and EhPTPA-over-expressing parasites had differential adherence to healthy and apoptotic cells, we measured adherence to staurosporine-treated apoptotic CHO cells. In agreement with our previous data, the adherence of control parasites to apoptotic CHO cells was approximately 40% diminished compared with healthy CHO cells (Fig. 4B) (Baxt et al., 2010). The adherence of EhPTPA-over-expressing parasites to apoptotic CHO cells was significantly reduced compared with healthy CHO cells, yet was comparable with the adherence of the control (Fig. 4B). Interestingly, the adherence of EhSIAF-over-expressing parasites to apoptotic CHO cells was not reduced, indicating that the increased adherence of EhSIAF-over-expressing parasites is observed on both healthy and apoptotic cells, similar to the role of PATMK on adhesion to erythrocytes (Boettner et al., 2008).

3.6. Over-expressing EhSIAF results in decreased parasitic cytotoxicity

Lectin-dependent binding of *E. histolytica* trophozoites to host cells is a requirement for parasite cytotoxicity (Petri et al., 1987; Saffer and Petri, 1991; Huston et al., 2000). The observed increased adherence properties of EhSIAF and EhPTPA-over-expressing parasites prompted us to investigate whether their cytotoxicity was also increased. Unexpectedly, the CHO cell monolayer destruction ability of EhSIAF-over-expressing parasites was reduced by approximately 50% compared with controls (P value < 0.003), whereas the cytotoxicity of EhPTPA-over-expressing transfectants was similar to control cells (Fig. 5A). Thus, EhSIAF-over-expressing parasites exhibit a complex phenotype with increased adherence but diminished cytotoxicity. This paradoxical phenotype hints that signaling events downstream of adhesion may be responsible for the decreased virulence phenotype or that over-expression of EhSIAF is resulting in a dominant-negative phenotype, at least for some of its functions.

3.7. Impaired motility of EhSIAF and EhPTPA-over-expressing transfectants

In order to penetrate and destroy colonic epithelium and travel to extra-intestinal sites of infection, parasite motility is required (Voigt and Guillen, 1999; Labruyere and Guillen, 2006). Nachin et al. (2005) reported the phenotypes of different mutants of *Escherichia coli* universal stress protein (UspA) which displayed increased adhesion to yeast cell surface and decreased motility on low-agar plates. The observed increased adherence properties of EhSIAF and EhPTPA-over-expressing parasites and the decreased cytotoxicity of EhSIAF-over-expressing parasites prompted us to further investigate whether their motility was also decreased. We monitored the ability of parasites to migrate through 8 μ m pores of the transwell inserts. Our data demonstrate that both EhSIAF and EhPTPA-over-expressing amoebae were strongly (approximately 80%) diminished compared with control parasites (P value < 0.000010) in their ability to migrate through the transwell inserts (Fig. 5B). The severe defect in transwell motility in both EhSIAF and EhPTPA-over-expressing amoebae may be a consequence of the increased adherence but future studies aimed at dissecting this are needed.

3.8. No significant changes are noted in phagocytosis of hRBCs

Another phenotype associated with amoebic virulence potential is the parasite's ability to ingest RBCs (Griffin, 1972). It has been shown that trophozoites attach to erythrocytes via the amoebic Gal/GalNAc lectin as well as PATMK (Petri et al., 1987; Boettner et al., 2008). Here we aimed to investigate whether EhPTPA and EhSIAF have an impact on

erythrophagocytosis, particularly whether EhSIAF-over-expressing parasites are decreased in their ability to ingest hRBCs, given the previously observed defect in cytotoxicity. Therefore hRBCs were co-incubated with trophozoites, followed by lysis of extracellular hRBCs and measurement of ingested erythrocytes by recording hemoglobin absorbance at 405 nm. Overall, there were no significant changes in phagocytosis of hRBCs noted in *E. histolytica* strains that over-expressed EhSIAF or EhPTPA compared with controls (Fig. 5C). We could not demonstrate for EhSIAF that its increased adhesion, reduced motility and reduced cytotoxicity resulted in a decreased ability to ingest hRBCs. However, it is possible that the detection of a small defect was not noted due to the inherent noisiness of the assay.

4. Discussion

We have characterized two amoebic proteins, EhSIAF and EhPTPA, whose corresponding genes exhibited higher transcript abundance in response to oxidative and nitrosative stresses. We generated transfectant trophozoite lines over-expressing these genes and characterized phenotypes associated with virulence (summarized in Table 2). We demonstrated that over-expression of each gene confers a survival advantage under oxidative stress, increases adherence to healthy host cells and decreases motility. Interestingly, over-expression of EhSIAF resulted in decreased virulence despite having an increased adherence phenotype. Overall, the data indicate that amoebic genes involved in the oxidative stress response can impact multiple phenotypes associated with amoebic pathogenesis.

Our previous work demonstrated that the response to oxidative and nitrosative stresses is modulated by a large and complex network of genes in *E. histolytica* (Vicente et al., 2009). These data were recently confirmed by another transcriptomics study with amoebae exposed to the nitrosative agent sodium nitroprusside (Santi-Rocca et al., 2012). The largest fraction of modulated genes encodes hypothetical proteins of unknown function, implying that the parasite's stress response remains largely unexplored. Given that *E. histolytica* faces oxidative and nitrosative stress upon host invasion, a significant fraction of the regulated genes is likely to be implicated in parasite virulence either directly or by increasing parasite resistance to such stresses.

Our results demonstrate that both EhSIAF and EhPTPA contribute to increased survival under hydrogen peroxide stress, supporting the results of the transcriptome analysis. EhPTPA is annotated as P-type ATPase. There are several reports confirming that P-type ATPases of such pathogens as *Cryptococcus neoformans* and *Candida albicans* are obviously involved in stress tolerance and virulence (Bates et al., 2005; Hu and Kronstad, 2009). As for EhSIAF, we see parallels to the *E. histolytica* thiol-specific antioxidant (TSA) protein (later identified as peroxiredoxin). TSA detoxifies hydrogen peroxide, is distributed through the surface and cytoplasm and co-localizes with the cytoplasmic domain of Gal/GalNAc lectin, suggesting sophisticated and robust parasite defense mechanisms against ROS (Bruchhaus et al., 1997; Poole et al., 1997; Hughes et al., 2003; Choi et al., 2005). Notably, when over-expressed in the avirulent strain, peroxiredoxin afforded increased resistance to ROS (Davis et al., 2006). Our data suggest that EhSIAF could act similarly to peroxiredoxin; future experiments on possible EhSIAF-Gal/GalNAc lectin interactions may elucidate such functions. Since direct scavenging of hydrogen peroxide by EhSIAF and EhPTPA is unlikely, we assume the presence of an indirect resistance mechanism, which needs further investigation.

Both EhSIAF and EhPTPA appear to have roles in amoebic adherence, a crucial step in parasite pathogenesis. Similarly, it has been shown that in *Campylobacter jejuni*, global regulator CsrA mediates the oxidative stress response and has additional roles in adherence to and invasion of intestinal epithelial cells (Fields and Thompson, 2008). Additionally, our

results imply that EhSIAF and EhPTPA may utilize different receptors and signaling pathways for amoebic adhesion since they have different adhesion capabilities to apoptotic host cells. Although adhesion, cell killing and phagocytosis of host cells by *E. histolytica* are sequential processes (Huston et al., 2003), our data demonstrate that the EhSIAF protein, despite promoting adhesion to host cells, reduces amoebic cytotoxicity and has no effect on erythrophagocytosis. Increased adherence resulting in decreased motility is one possible explanation for the reduced virulence phenotype of the EhSIAF protein. A parallel observation has been reported for *C. jejuni* CsrA which, in addition to playing a role in the oxidative stress response, also regulates host cell adherence while repressing host cell invasion (Fields and Thompson, 2008). Alternately, localization of EhSIAF in the proximity of internal membranes and vesicles suggests that EhSIAF may be involved in various signaling events, which could be negatively impacted by the over-expression of EhSIAF. The paradoxical phenotype hints that signaling events downstream of adhesion (such as vesicle trafficking and release of amoebapores) may be responsible for the decreased virulence phenotype and that over-expression of EhSIAF is resulting in a dominant-negative phenotype, at least for some of its functions. Our findings also imply that EhSIAF and EhPTPA might employ different cytotoxicity-related signaling pathways downstream of adhesion, because despite the fact that the phenotype of EhPTPA-over-expressing parasites showed increased adherence to healthy mammalian cells and was reduced in its motility, cytotoxicity was not affected.

Our studies reveal intricate phenotypes for genes involved in the oxidative stress response and illustrate a close correlation between the response to oxidative stress and virulence. Future studies focusing on elucidating the differential mechanisms of action and biological significance in EhSIAF and EhPTPA proteins of invasive amoebiasis are needed.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Highlights

- Studies of *Entamoeba histolytica* proteins called SIAF and PTPA whose mRNA level was upregulated upon oxidative stress.
- Stable transfectant strains expressing these genes were obtained and phenotypes associated with virulence characterized.
- EhSIAF and EhPTPA are specifically involved in resistance to oxidative stress and have a role in amoebic adherence.
- Both proteins are likely to be involved in stress adaptation in the context of *Entamoeba*-host interaction.

Fig. 1.

Expression of EhSIAF in *Entamoeba histolytica* trophozoites stably transfected with a N-terminal Myc-tagged protein. (A, B) Western blot analysis of EhSIAF under basal conditions and when exposed to 1 mM hydrogen peroxide (H_2O_2) (A) or 200 μ M dipropyleneetriamine (DPTA)-NONOate (B). Over-expression of Myc-tagged EhSIAF resulted in positive signal at the predicted size of 16 kDa, and two extra bands corresponding to potential dimer (32 kDa) and trimer (48 kDa) forms. No significant changes in protein levels were noted under either stress condition. Blots were probed with antibody to Myc- tag (1:1,000) and to α -Actin (1:1,000) as a loading control. Signal detection was performed using enhanced chemiluminescence. (C) Immunofluorescence assays were performed on permeabilized and non-permeabilized parasites over-expressing Myc-tagged EhSIAF. Fixed parasites were stained with primary antibodies α -Myc (1:250) and α -surface carbohydrate-binding lectin (Hgl) (1:50), followed by incubation with fluorescent secondary α -mouse Alexa 594 or Alexa 488 antibodies (1:1,000). The localization of EhSIAF protein on permeabilized amoebae appears to be predominantly cytoplasmic. Some signal intensity was also observed close to cellular membranes. Due to the lack of the signal co-localization with GalNAc lectin on non-permeabilized parasites, it appears that EhSIAF protein was located on the inner leaflet of the plasma membrane. Imaging was performed on a Leica CTR6000 microscope, using a BD CARVII confocal unit. Scale bar = 10 μ m.

Fig. 2.

Expression of EhPTPA in *Entamoeba histolytica* trophozoites stably transfected with a C-terminal Myc tagged protein. (A) Western blot analysis of EhPTPA under basal conditions and when exposed to 1 mM hydrogen peroxide (H_2O_2) or to 200 μ M dipropyleneetriamine (DPTA)-NONOate. Over-expression of Myc-tagged EhPTPA resulted in positive signal only upon exposure to oxidative or nitrosative stress, with smeary bands at the predicted size of a monomer (120 kDa) and as a potential dimer form (240 kDa). Blots were probed with antibody to Myc- tag (1:1,000) and to α -Actin (1:1,000) as a loading control. Signal detection was performed using enhanced chemiluminescence. (B) Immunofluorescence assays were performed on fixed and permeabilized parasites over-expressing Myc-tagged EhPTPA. Parasites were stained with primary antibody α -Myc (1:250) followed by incubation with fluorescent secondary antibody α -mouse Alexa 594. Myc-tagged EhPTPA

is mainly localized on the internal vesicles in the cytoplasm. Imaging was performed on a Leica CTR6000 microscope, using a BD CARVII confocal unit. Scale bar = 10 μ m. DIC, differential interference contrast image.

Fig. 3.

Entamoeba histolytica HM-1: IMSS parasites over-expressing EhSIAF and EhPTPA had increased resistance towards hydrogen peroxide treatment. Parasites were treated with 1 mM hydrogen peroxide for 1 h at 37°C and stained with 0.2% trypan blue to assess cell viability. Data are expressed in arbitrary units with control survival normalized to 1.0. The average of five experiments and S.D. are shown. **P*value < 0.013 (EhSIAF), #*P*value < 0.019 (EhPTPA). Control vector contains luciferase gene.

Fig. 4.

Over-expression of EhSIAF in *Entamoeba histolytica* HM-1: IMSS parasites results in increased adherence to both healthy and apoptotic Chinese hamster ovary (CHO) cells, whereas over-expression of EhPTPA results in increased adherence only to healthy CHO cells. (A) Adhesion was measured with a CHO cell rosette assay. Parasites were mixed with CHO cells at 4°C for 1 h, and parasites with three or more CHO cells attached were counted as positive. EhSIAF and EhPTPA-over-expressing parasites displayed an increased adherence to healthy CHO cells compared with controls. The control cell lines over-express the luciferase protein. Data are expressed as the percentage of the control strain adherence levels and represent the average of five experiments, each with triplicates. S.D.s are shown, **P* value < 0.01 for control compared with EhSIAF and EhPTPA. (B) CHO cells were treated either with 1 μM staurosporine aglycone or with 1 μM DMSO for 1 h at 4°C, and a CHO cell rosette assay was performed. Over-expression of EhSIAF resulted in increased adherence to apoptotic CHO cells, whereas over-expression of EhPTPA resulted in increased adherence only to healthy CHO cells. Data are expressed as the percentage of control DMSO-treated levels and are the average of three independent experiments. S.D.s are shown. **P* value < 0.01 for control (DMSO) compared with EhPTPA (DMSO); †*P* value < 0.004 control (staurosporine) compared with EhSIAF (staurosporine). The control cell lines over-express the luciferase protein.

Fig. 5.

Over-expression of EhSIAF in *Entamoeba histolytica* HM-1: IMSS parasites results in reduced cytotoxicity and motility but has no effect on erythrophagocytosis. (A) Cytotoxicity was measured by placing a total of 0.5×10^5 trophozoites on a confluent chinese hamster ovary (CHO) cell monolayer for 2 h at 37°C, followed by fixation, staining with 0.1% methylene blue, dye extraction and spectrophotometric determinations at 650 nm. Over-expression of EhSIAF protein resulted in parasites with decreased cytotoxicity, while the over-expression of EhPTPA protein had no effect on parasites' cytotoxicity and was comparable with the control level. The results represent the means and S.D.s of four independent experiments and are expressed as the percentage of the control strain destruction level. * P value < 0.003 control compared with EhSIAF. (B) Motility was measured by assessing the number of parasites that migrated through the transwell chamber. A total of 1.5×10^5 parasites were added to the upper chamber of a transwell system and allowed to migrate into the lower chamber for 3 h at 37°C. Over-expression of both EhSIAF and EhPTPA resulted in parasites with strongly diminished migration properties through the transwell compared with the control. The average of three independent experiments is shown with S.D. Data are shown as a percentage of control strain motility. * P value < 0.00003 for control compared with EhSIAF and P value < 0.00009 for control compared with EhPTPA. (C) Erythrophagocytosis was measured by incubation 1×10^5 trophozoites with 1×10^8 human red blood cells (hRBC) for 15 min at 37°C, followed by lysis of extracellular hRBC and measurement of ingested erythrocytes at 405 nm. No significant changes in phagocytosis of hRBCs were noted in *E. histolytica* strains that over-expressed EhSIAF or EhPTPA compared with control. The results represent the means and S.D.s of four independent experiments and are expressed as the percentage of the control strain erythrophagocytosis level. The control cell lines over-express the luciferase protein.

Summary of gene expression data for *Entamoeba histolytica* EhSIAF and EhPTPA genes under oxidative and nitrosative stress conditions. The probe ID, *E. histolytica* identification (EHI) accession number, protein homology, fold change and *P* value under oxidative and nitrosative stress conditions are shown. EhSIAF and EhPTPA were differentially upregulated upon oxidative (EhSIAF, 15-fold and EhPTPA, 2.3-fold) and nitrosative (EhSIAF, 13.5-fold and EhPTPA, 2.6-fold) stress in *Entamoeba histolytica* HM-1:IMSS parasites. Numbers between brackets represent the average of three independent array data experiments and show the protein basal expression level (wild type conditions) and the protein expression level after stress with 1 mM hydrogen peroxide or 200 μ M dipropyleneetriamine (DPTA)-NONOate (1 h at 37°C). Microarray data were adapted from Vicente et al. (2009).

Table 1

Probe ID	EHI accession number	Annotation	Fold change (oxidative stress)	<i>P</i> value	Fold change (nitrosative stress)	<i>P</i> value
EhSIAF	EHL_056680	Hypothetical protein	15 (0.24 – 2.71)	2.52E-02	13.5 (0.24 – 2.38)	3.26E-02
EhPTPA	EHL_188210	P-type ATPase	2.3 (1.18–2.74)	6.56E-03	2.6 (1.18–3.06)	5.46E-04

Summary of phenotypes observed in *Entamoeba histolytica* HM-1: IMSS parasites over-expressing EhSIAF and EhPTPA genes.

Table 2

Phenotype	EhSIAF	EhPTPA
Protein level upon exposure to oxidative stress	~	↑
Protein level upon exposure to nitrosative stress	~	↑
Resistance to oxidative stress	↑	↑
Adherence to healthy CHO cells	↑	↑
Adherence to apoptotic CHO cells	↑	~
Cytotoxicity	↓	~
Motility	↓	↓
Erythrophagocytosis	~	~

CHO, chinese hamster ovary; no change, ~; increase, ↑; decrease, ↓.