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An unusual surface peroxiredoxin protects invasive *Entamoeba histolytica* from oxidant attack

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Abstract

Peroxiredoxins are an important class of antioxidant enzymes found from Archaea to humans, which reduce and thereby detoxify peroxides and peroxynitrites. The major thiol-containing surface antigen of the invasive ameba, *Entamoeba histolytica*, is a peroxiredoxin and is likely to be important during the transition from the anaerobic environment of the large intestine to human tissues. The closely related species, *Entamoeba dispar*, is incapable of invasion and more sensitive to hydrogen peroxide, yet also has a peroxiredoxin. We cloned and expressed the two active recombinant enzymes and found that their activity was similar by a fluorometric stopped-flow assay, giving a K_m of <10 μM for hydrogen peroxide. Three monoclonal antibodies produced to recombinant *E. histolytica* peroxiredoxin cross-reacted with *Entamoeba dispar*. *E. histolytica* contains as much as 50 times more peroxiredoxin than *E. dispar* as demonstrated by a sensitive capture ELISA. In addition, the peroxiredoxin is present largely on the outer surface of the cell, in contrast to *E. dispar*. This unusual peroxiredoxin localizes to the site of parasite–host cell contact where it can effectively counteract oxidants generated by host cells, thus facilitating invasion.

Keywords: *Entamoeba histolytica*; Peroxiredoxin; Antioxidant; Amebiasis

1. Introduction

Entamoeba histolytica is a protozoan parasite, which causes life-threatening amebic colitis and liver abscesses in tropical and subtropical countries. It is third only to malaria and schistosomiasis as a cause of death from parasitic diseases [1]. *E. histolytica* trophozoites live in an anaerobic or microaerobic environment in the large intestine where they are exposed to a low concentration of oxidants from the metabolism of surrounding bacteria. However, once trophozoites invade the tissue, they must survive both a higher ambient oxygen environment and exposure to reactive oxygen species generated by the primary protective effector cells, including neutrophils and macrophages.

A number of strategies have been developed by prokaryotic and eukaryotic organisms to protect against oxygen toxicity. These include enzymes that decompose peroxides and superoxide anions, small molecule antioxidants such as Vitamins E and C, and thiols, which scavenge transient free radicals (reviewed in [2]). Three main enzymes have been identified which may serve as antioxidants in *E. histolytica*: an iron-containing superoxide dismutase (Fe-SOD) which catalyzes the conversion of superoxide to O_2 and hydrogen peroxide (H_2O_2) [3–4], a flavin reductase (NADPH:flavin oxidoreductase) which can also reduce O_2 to H_2O_2 [5], and a thiol-specific antioxidant (TSA) or peroxiredoxin, which has both peroxidase and antioxidant activities [6–9]. Earlier studies identified H_2O_2 as the primary lethal oxygen

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derivative for *Entamoeba* [3,10], and a "highly virulent" strain of *E. histolytica* was found to be less susceptible to H_2O_2 than an attenuated strain, which had lost its virulence in prolonged culture [3,10]. A more biologically relevant comparison would be between potentially invasive *E. histolytica* and the closely related non-invasive *E. dispar.* These organisms are morphologically identical and live in the same environmental niche, but can be differentiated by clinical symptoms of the patient, isoenzymes [11], surface antigens [12–14] and PCR [15–16]. *E. histolytica* and *E. dispar* are now recognized as different species [1], and only *E. histolytica* can invade the human host.

The peroxiredoxin of *E. histolytica*, a thiol-rich 29-kDa surface antigen, has peroxidase activity and is likely to be a key protein in protection from H_2O_2 generated from internal and environmental oxidative stress [6,9]. The activity of the other two antioxidants, FeSOD and flavin reductase, results in the formation of H_2O_2, which cannot be detoxified without catalase in *Entamoeba*. Genes encoding homologues to the *E. histolytica* peroxiredoxin have recently been cloned from *E. dispar* [17] and *Entamoeba moshkovskii* [18]. Some epitope differences in the expressed proteins of *E. histolytica* and *E. dispar* must exist as a number of monoclonal antibodies react only with the *E. histolytica* peroxiredoxin [12–13]. The peroxiredoxin is released during invasive amebiasis as the majority of patients develop specific antibodies [19]. Moreover, immunization with recombinant antigen protected gerbils from liver abscesses [20]. A peroxiredoxin of *Leishmania major* was also shown to be protective when given as part of a DNA vaccine to mice [21].

We cloned and compared the active recombinant peroxiredoxins and now report critical differences between the antioxidant activities of *E. histolytica* and *E. dispar. E. histolytica* is characterized by: (1) increased resistance to H_2O_2-mediated killing, (2) more peroxiredoxin and (3) a primarily surface-localized peroxiredoxin, in contrast to *E. dispar.* The surface-localized peroxiredoxin of *E. histolytica* is an important protective factor against oxidative killing and thus may facilitate invasion of the host.

2. Materials and methods

2.1. Amebae cultures

Axenic *E. histolytica* strain HM-1:IMSS and monoxenic *E. dispar* strain SAW 1734 were cultivated in TYI-S-33 medium [22] supplemented with 15% bovine calf serum (Irvine Scientific, Irvine, CA). For long-term maintenance, *E. dispar* cultures were supplemented with *Crithidia fasciculata.* Clinical isolates were maintained in Robinson's BRS medium with added *Escherichia coli* [23] and subcultured three times a week. Strains were identified as *E. histolytica* or *E. dispar* based on the clinical presentation of the patient, zymodemes [11] and PCR amplification of rRNA sequences [15].

2.2. H_2O_2 killing assay

Clinical isolates of *E. histolytica* and *E. dispar* grown in BRS media were harvested by completely removing the existing media, adding 50 ml of fresh BRS, and chilling the flasks briefly on ice. Following centrifugation at $700 \times g$ for 10 min, the cell pellet was resuspended in Dulbecco's modified Eagle's medium (DME; Invitrogen, San Diego, CA) supplemented with 70 mg L-cysteine and 135 mg ascorbic acid/100 ml (DME-CH, pH 7.4) to a final concentration of 1×10^6 cells/ml. Trophozoites of *E. histolytica* and *E. dispar* isolates were incubated with 0–10 mM H_2O_2 at 37 °C with gentle rocking for 1 h. At timed intervals, aliquots were removed and cell viability was determined by trypan blue exclusion. Statistics were determined by Student's *t*-test.

2.3. Antibodies

Monoclonal antibodies were produced in mice to the p47 clone of the peroxiredoxin of *E. histolytica* expressed in *E. coli* as a glutathione fusion protein as previously described [24]. The epitopes of the monoclonal antibodies were mapped using truncated fusion proteins expressed from the p47 cDNA cut with *Dra*I, *Hin*f1 or *Sau*3A1 and detected by immunoblots. The reactivity of the monoclonal antibodies against *E. histolytica* and *E. dispar* was determined by immunoblots and ELISA using lysates of clinical strains. FP-10 was directly labeled with horseradish peroxidase using EZ-Link Plus Activated Peroxidase kit (Pierce). Monospecific antibodies were produced in mice by immunization with the full-length glutathione fusion protein [24].

2.4. Cloning of peroxiredoxin genes

Genomic DNA was isolated from purified nuclei of *E. histolytica* axenic strain HM-1:IMSS and clinical *E. dispar* isolate SD119 using the DEPC-Triton X-100 method [25]. Briefly, approximately 5×10^6 amebae were lysed in 40 ml of Triton X-100-RSB buffer (10 mM Tris–HCl pH 7.4, 10 mM NaCl, 5 mM $MgCl_2$, 1% Triton X-100, 0.04% DEPC). The resulting nuclei were pelleted by low speed centrifugation and the DNA was purified using the DNeasy Tissue kit (Qiagen, Valencia, CA) following treatment with Proteinase K and RNase A.

The sequence of the full-length *E. dispar* peroxiredoxin gene (732 bp) was obtained by amplification of genomic DNA from clinical isolate SD119. The complete peroxiredoxin genes from *E. histolytica* and *E. dispar* were amplified from genomic DNA by PCR using oligonucleotide primer pairs derived from previously reported sequences [17,26], and cloned into pBAD/Thio-TOPO vector (Invitrogen) (*E. histolytica*: 5′ primer EH29TOPO5 = ATGAAGAGA-AGAAAAATTATT, 3′ primer EH29-TOPO3 = ATGTGCTGTTAA-ATATTTCTT) (*E. dispar*: 5′ primer = ATGTCCTGCA-ATCAACAAAAA, 3′ primer = ATGTGT-TGTTAAATATTTCTT). They were transformed

into INVaF′ chemically competent cells (Invitrogen) for recombinant expression.

2.5. Expression and purification of recombinant proteins

Expression of the recombinant peroxiredoxins was induced with 0.02% arabinose at 37 °C overnight. After centrifugation, the transformed bacteria were lysed in CelLytic B II (5 ml/g cell paste, Sigma) with deoxyribonuclease I (5 μg/ml, Promega) for 15 min at room temperature, sonicated six times each for 15 s and centrifuged at 25,000 × *g* for 15 min. The recombinant protein contained a thioredoxin fusion on the N-terminus and a 6-His tag on the C-terminus, and was purified by metal chelation chromatography using Ni–NTA spin columns (Qiagen), followed by immunoaffinity chromatography, using two monoclonal antibodies (FP8 and FP10) coupled to cyanogen bromide-activated Sepharose 4B (Amersham Pharmacia Biotech, Piscataway, NJ) [8]. *E. coli* thioredoxin was expressed and purified as previously described [27].

2.6. Peroxidase activity

Peroxidase activity of the purified recombinant enzymes was measured by a very sensitive fluorometric assay monitoring the decrease in tryptophan fluorescence of reduced thioredoxin (Trx) as it is oxidized during turnover with the peroxiredoxin and peroxides [28]. Details of the assay procedure were essentially the same as those described for the *Clostridium pasteurianum* peroxiredoxin system [29]. Briefly, 0.08–0.3 μM (final concentrations after mixing) of the peroxiredoxin plus the peroxide of choice (generally at 500 μM) in one syringe was mixed with prereduced *E. coli* Trx (5–20 μM) in the other syringe on an Applied Photophysics DX.17 MV stopped-flow spectrofluorometer thermostated at 25 °C. The buffer in both syringes contained 50 mM potassium phosphate at pH 7.0, 0.5 mM EDTA and 100 mM ammonium sulfate. The change in fluorescence over time (excitation at 280 nm, emission at 90° recorded using a 320 nm filter) was recorded and converted to micromolar Trx oxidized per second following calibration with known concentrations of reduced and oxidized Trx. Peroxiredoxin concentrations were determined by densitometry of samples on SDS polyacrylamide gels using Quantity One software (Bio-Rad, Hercules, CA) as described previously [30] to ensure that only the bands corresponding to the recombinant peroxiredoxin were quantitated.

2.7. Quantification of peroxiredoxin by ELISA and immunoblots

The amount of peroxiredoxin in cell lysates of axenic strains and clinical isolates of *E. histolytica* and *E. dispar* was determined by a sensitive capture ELISA. Trophozoites were washed three times in PBS, resuspended at a concentration of 1×10^6 cells/ml in 50 mM Tris, 2 mM EDTA, 0.1% Trition X-100, pH 7.5 containing proteinase inhibitor cocktail (Complete, Roche, Mannheim, Germany) and E-64 (20 μM, Roche). After two cycles of freeze/thawing, the lysates were standardized by cell number and protein for ELISAs and immunoblots.

ELISA plates were coated with monoclonal antibodies FP16 and FP21 (250 ng each) allowed to bind overnight at 4 °C, and blocked with 5% BSA in PBS for 2 h. Cell lysates (4×10^3–10^5 cells/ml or 350–9200 ng protein in PBS + inhibitors) were incubated in wells for 1 h, washed in PBS-Tween, reacted with horseradish peroxidase-labeled FP10 (1:50 dilution in PBS, 0.1% Tween), developed with SureBlue Reserve™ TMB (KPL) for 5 min, the reaction stopped by 1N HCl and the absorbance read at 450 nm by spectrophotometer. Recombinant peroxiredoxins of *E. histolytica* and *E. dispar* were used to generate a standard curve. Student's *t*-test was performed to determine the statistical significance.

Immunoblots were made by electrophoresing 0.5–5.0 μg of *E. histolytica* or *E. dispar* lysates by 12% SDS-PAGE under reducing conditions, probing with a mouse monospecific antibody (1:500 dilution) and detecting with goat anti-mouse IgG horseradish peroxidase (Zymed) and SuperSignal (Pierce) TMB (KPL). The monospecific antibodies react to both denatured *E. histolytica* and *E. dispar* peroxiredoxins [24]. The digitized image was taken with Gel Doc (BioRad) and Densitometry performed with BioRad Quantity One.

2.8. Confocal microscopy

Trophozoites were washed twice in PBS, pH 7.4, fixed in 10% formalin for 15 min at room temperature and blocked with 3% BSA in PBS or permeabilized in 0.1% Triton X-100 in PBS for 10 min before blocking. They were incubated with purified monoclonal antibody, which reacted with both the *E. histolytica* and *E. dispar* peroxiredoxin (FP10, 5 μg/ml), and antibody was detected with Alexa 488-conjugated goat anti-mouse antibody (1:500 dilution, Molecular Probes, Eugene, OR). The samples were imaged with a Zeiss 510 laser scanning confocal microscope and an argon/krypton laser. Image analysis was performed using LSM and Adobe Photoshop software. Negative controls include isotype matched antibodies and the conjugates alone.

3. Results

3.1. Susceptibility to H_2O_2 killing

We first compared the sensitivity of clinical isolates of *E. histolytica* and *E. dispar* to H_2O_2. Both species of amebae were killed by H_2O_2 in a time- and dose-dependent manner; however, *E. dispar* was significantly more susceptible to H_2O_2 than *E. histolytica* (Fig. 1).

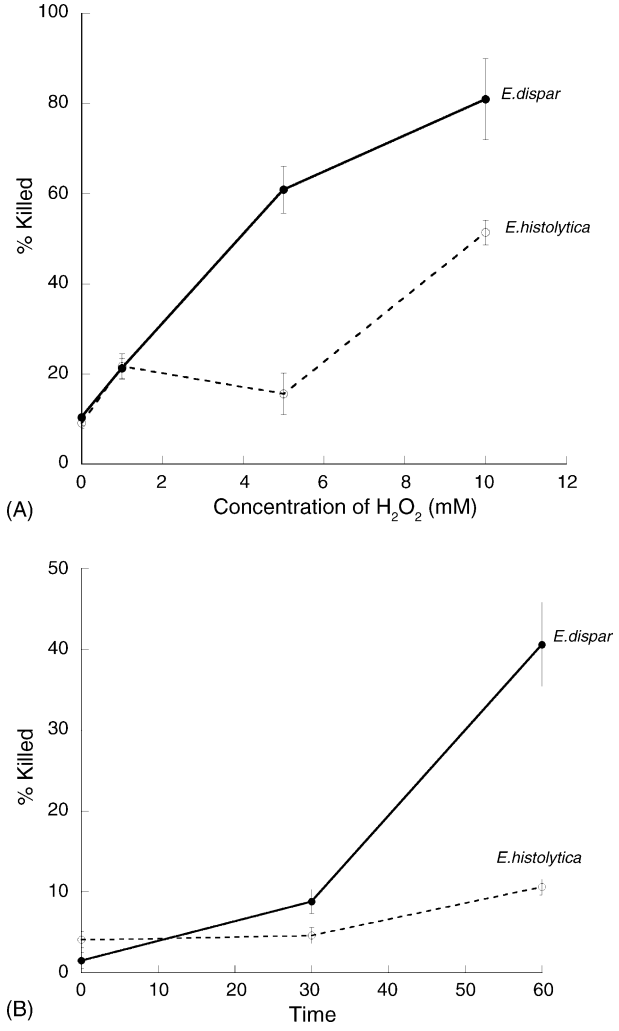

Fig. 1. Susceptibility of clinical isolates of *E. histolytica* and *E. dispar* to hydrogen peroxide killing. (A) Trophozoites were incubated in DME-CH with 0–10 mM H_2O_2 for 1 h at 37 °C and the viability assessed by trypan blue exclusion. (B) Trophozoites were incubated with 5 mM H_2O_2 and aliquots removed at timed intervals to determine viability. Data represent the mean and standard error of at least three independent experiments with at least three different strains performed in triplicate. The differences in killing were significant at all time points at $p < 0.01$ (Student's *t*-test).

3.2. Cloning of the peroxiredoxin genes

The complete peroxiredoxin gene of *E. histolytica* had previously been cloned [12]. Using primers based on conserved sequences of peroxiredoxin genes, we amplified a 325 bp fragment from *E. dispar* genomic DNA, and the full-length sequence (732 bp) by amplification from an *E. dispar* library (kind gift of Dr. Barbara Mann).

We also obtained the nucleotide sequence from a clinical isolate of *E. dispar* (SD119) and compared it to *E. histolytica* and the previously reported sequence from *E. dispar* [17]. Like Tachibana and Cheng, we found that our clone had a number of single nucleotide substitutions. The consensus sequence encoded a polypeptide of 243 amino acids, and comparison of nucleotide sequences with the sequence of *E. dispar* [17] revealed a substitution of single nucleotides in 24 positions, which resulted in 12 amino acid changes (Fig. 2). The sequence also showed 75 single nucleotide changes when compared to that of *E. histolytica* [24], causing amino acid changes in 16 sites.

A partial sequence encoding a second potential peroxidoxin was identified through the *E. histolytica* genome project; however, it contained multiple stop codons and was not transcribed (data not shown).

3.3. Expression and activity measurements of recombinant peroxiredoxin

To evaluate the peroxidase activity of recombinant peroxiredoxins, primers were designed to amplify the entire genes encoding peroxiredoxins of *E. histolytica* and *E. dispar*. The genes were subcloned into the pBAD/Thio-TOPO expression vector driven by the *ara*BAD promoter (P_{BAD}). The resulting thioredoxin fusion proteins were purified by nickel affinity chromatography using the poly-histidine tag and immunoaffinity chromatography was performed with two monoclonal antibodies (FP8 and FP10) to remove any contaminants. Successful purification of the recombinant protein by this two-step procedure was demonstrated by SDS-PAGE with a single 45-kDa band, consistent with the 29-kDa peroxiredoxin and 16-kDa fused thioredoxin protein (Fig. 3).

The peroxidase activity of the recombinant enzymes was measured by a fluorometric stopped-flow assay in a system containing reduced *E. coli* Trx, limiting amounts of the peroxiredoxin and the peroxide substrate. Using this assay, the activities of the *E. histolytica* and *E. dispar* enzymes toward peroxiredoxin were found to be less than two-fold different based on the slopes of the lines (Fig. 4A), with the *E. dispar* enzyme exhibiting a somewhat greater level of activity. More in depth kinetic studies with the *E. dispar* enzyme indicated that hydrogen peroxide was the best substrate for these enzymes, although the alkyl and aryl hydroperoxides gave rates at 60, 87 and 85% of that with hydrogen peroxide for cumene hydroperoxide, *t*-butyl hydroperoxide and ethyl hydroperoxide, respectively (Fig. 4B). Activities with hydrogen peroxide increased linearly with Trx concentrations up to 20 μM (data not shown), indicating a non-saturable, bimolecular interaction (or saturable only at very high, non-physiological concentrations) with this reductant. Both saturable and non-saturable kinetics with their electron donors have been observed for peroxiredoxins from other organisms [31]; the second order rate constant at about $1 \times 10^5\ M^{-1}\ s^{-1}$, is also identical with that determined for the *Helicobacter pylori* thioredoxin-dependent peroxiredoxin [32]. This sensitive, peroxiredoxin-dependent assay also revealed a very low K_m value (<10 μM) for hydrogen peroxide, indicating high reactivity of these enzymes toward peroxides at even very low peroxide concentrations.

```
E. dispar SD119     ATGTCCTGCAATCAACAAAAAGAATGTTGTAAGAAAGAGTGCTGTAAGAAAGAATGTCAA  60
E. dispar           --------T----------------------------A--TCAAG-----------TGT
E. histolytica                            ---.-C-.T-C--...-.-AAC--.-----G---TGT

E. dispar SD119     GAGAAAGAATGTTGTAAAGAGTATTGTAAGAAAGAATGTTGTTGTCCAAGAATTAAAGCA  120
E. dispar           A-AG-GT-T-------------------------------------------------
E. histolytica      A-A---------CAAG-GA-AG-A---TGT----------------------A------

E. dispar SD119     TTTAAGAAATTTATGAACACATTTGAAAAAGCACAAATTGGAAAAGAAGCGCCAGAATTT  180
E. dispar           ---------------------------------------------A---------
E. histolytica      --------------A-----------------------------------A---------

E. dispar SD119     AAAGCACCAGCATATTGTCCATGTGGTTCAATCAAAGAAATTGATATTAATGAATATAAA  240
E. dispar           -------A-------------------------------------------------
E. histolytica      ------------------------------------G------------------G-

E. dispar SD119     GGGAAATATGTTGTGTTATTATTTTATCCATTGGATTGGACATTTGTTTGTCCAACAGAA  300
E. dispar           -------------------------------------------------------------
E. histolytica      --A-----------A--G--G-----------------------------------------

E. dispar SD119     ATGATTGGATATAGTGAAGTTGCAGGACAATTGAAAGAAATCAATTGTGAAGTTATTGGA  360
E. dispar           -------------------------------------------------------------
E. histolytica      ------------------C-----------------------------------------

E. dispar SD119     GTGAGTGTTGATTCAGTTTATTGTCATCAAGCATGGTGTGAAGCAGATAAAAGTAAAGGA  420
E. dispar           -------------------------------------------------------------
E. histolytica      --------A-------------------------------------------------

E. dispar SD119     GGAGTAGGAAAATTAGGATTCCCATTAGTATCAGATATTAAAAGATGTATTTCAATTAAA  480
E. dispar           -------------------------------------------------------------
E. histolytica      -----------G--GAC-----------------------G-----C-----T--C---

E. dispar SD119     TATGGAATGTTAAATGTAGAAACAGGAGTTTCAAGAAGAAGATATGTCATTATTGATGAT  540
E. dispar           ----------------------------------------G--------------------
E. histolytica      ------------------C---G-----A--G--------G----------C-----C---

E. dispar SD119     AAAGGAAAAGTAAGATACATTCGAATGAACGATGATGGAATTGGAAGATCAACAGAAGAA  600
E. dispar           ----------------------A------T-------------------------------
E. histolytica      ----------------------A------T------------------------G------

E. dispar SD119     ACTATTAGAATAGTTAAAGCAATTCAATTTAGTGAAGAACATGGAACAGCTTGTCCAATC  660
E. dispar           ------------------------------------------G---T----------
E. histolytica      --A--C--------G-------------C-----T---------G---T-------C--

E. dispar SD119     AATTGGAAAGCAGGGAAAGACACAATTGAAACAACACCAGAAGGAATCAAGAAATATTTA  720
E. dispar           ------------------------------C-------------------------------
E. histolytica      ---------C----C--------C------C----------T-----T------------

E. dispar SD119     ACAACACATTAA                                                 732
E. dispar           ------------
E. histolytica      ---G--------
```

(A)

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E. dispar SD119     MSCNQQKECCKKECCKKECQEKECCKEYCKKECCCPRIKAFKKFMNTFEKAQIGKEAPEF  60
E. dispar           --------------QE---CKEY-------------------------------------
E. histolytica                Q--------------.....-------------------------------
                                                 *
E. dispar SD119     KAPAYCPCGSIKEIDINEYKGKYVVLLFYPLDWTFVCPTEMIGYSEVAGQLKEINCEVIG  120
E. dispar           --Q-------------------------------------------------------
E. histolytica      -------------------------------------------------------------

E. dispar SD119     VSVDSVYCHQAWCEADKSKGGVGKLGFPLVSDIKRCISIKYGMLNVETGVSRRRYVIIDD  180
E. dispar           ---------------------------------------------------G------
E. histolytica      ------------------------T--------------------A-IA--G------
                                                    *
E. dispar SD119     KGKVRYIRMNDDGIGRSTEETIRIVKAIQFSEEHGTACPINWKAGKDTIETTPEGIKKYL  240
E. dispar           -------Q---------------------------AV-------------P---------
E. histolytica      -------Q----------------------D---AV------P------P--D------

E. dispar SD119     TTH                                                          243
E. dispar           ---
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(B)

Fig. 2. Comparison of nucleotide (A) and deduced amino acid (B) sequences of peroxiredoxin from clinical isolate of *E. dispar* (SD119, top line) with monoxenic *E. dispar* strain (middle line, [17]) and *E. histolytica* (bottom line, [9]). The sequences were aligned with MacVector 3.5. Identical nucleotide or amino acid residues are indicated by dashes, and the dots designate gaps in the sequence alignment. Two cysteine residues essential for the peroxiredoxin activity are indicated by asterisks.

3.4. Quantification of peroxiredoxin in E. histolytica and E. dispar

Monoclonal antibodies FP-10, FP16 and FP-21 [24] were found to recognize different epitopes of the native peroxiredoxins of *E. histolytica* and *E. dispar* (manuscript in preparation). FP-21 had previously been shown to inhibit peroxiredoxin activity while FP-10 and FP-16 did not [6]. In a sensitive capture ELISA, *E. histolytica* lysates had >50 times more peroxiredoxin than *E. dispar* whether standardized to

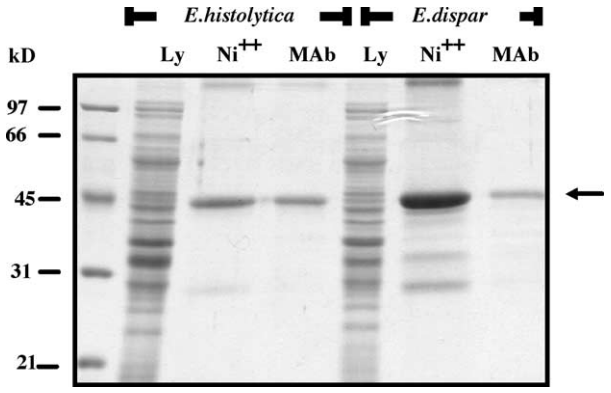

Fig. 3. Purification of recombinant peroxiredoxins of *E. histolytica* and *E. dispar* by nickel and immunoaffinity chromatography (SDS-PAGE under reducing conditions). Lane 1, lysate of *E. coli* transformed with Eh29kd of *E. histolytica* (Ly); lane 2, partially purified fraction of *E. histolytica* after purification over Ni–NTA agarose (Ni^{2+}); lane 3, recombinant peroxiredoxin of *E. histolytica* finally purified by immunoaffinity chromatography (MAb); lane 4, lysate of *E. coli* transformed with Ed29kd of *E. dispar* (Ly); lane 5, partially purified fraction of *E. dispar* after purification over Ni–NTA agarose (Ni^{2+}); lane 6, recombinant peroxiredoxin of *E. dispar* finally purified by immunoaffinity chromatography (MAb). Arrow points to purified recombinant peroxiredoxin.

amount of protein (Fig. 5, $p < 0.001$) or cell number (data not shown). These findings were also confirmed by immunoblots reacted with a monospecific mouse antibody (Fig. 6). A second band (~60 kDa), representing a dimer, could be detected on the gels of *E. histolytica*, but not *E. dispar*. *E. histolytica* lysates had 4–7× as much peroxiredoxin proteins as *E. dispar* by densitometry of the bands. The smaller difference most likely represents the lower sensitivity of immunoblots, or could also be due to the solution dynamics of the capture assay.

3.5. Confocal microscopy

Localization of the peroxiredoxin was determined by confocal microscopy in Triton X-100 permeabilized and unpermeabilized trophozoites of *E. histolytica* and *E. dispar*. Using a monoclonal antibody (FP10) which cross-reacted with the peroxiredoxin of both species, we found that the enzyme was primarily surface localized in *E. histolytica* whether the trophozoites were solubilized or not, in contrast to cytoplasmic distribution in *E. dispar* (Fig. 7). When trophozoites were not solubilized with detergent, there was no reactivity with *E. dispar* strains, indicating that the peroxiredoxin is exposed on the surface of *E. histolytica*, but not *E. dispar* (data not shown).

4. Discussion

The peroxiredoxin is the major thiol-containing surface antigen of *E. histolytica* [8]. It belongs to a widely dispersed group of proteins found from Archaea to plants to humans with peroxidase and antioxidant activity, termed

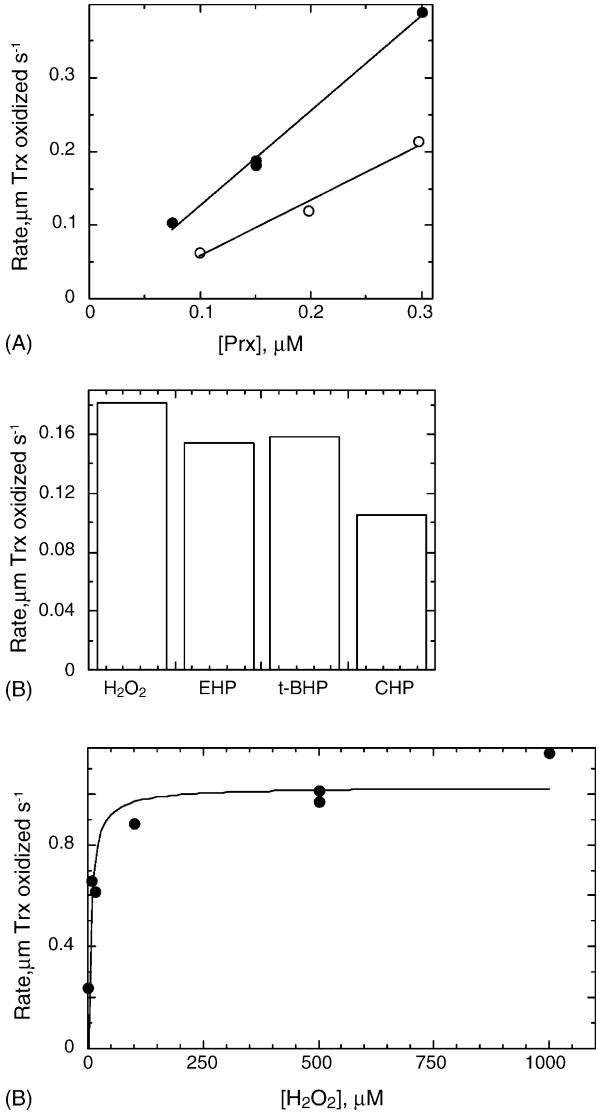

Fig. 4. Catalytic assays of *E. histolytica* and *E. dispar* peroxiredoxins. (A) Each of the peroxiredoxins were varied from 0.08 to 0.3 μM (final concentrations after mixing) and assayed on a stopped-flow spectrofluorometer at 25 °C, measuring the decrease in fluorescence (excitation at 280, 320 nm filter for emission) as excess reduced thioredoxin (10 μM) is oxidized during peroxiredoxin turnover with 500 μM hydrogen peroxide. Rates of fluorescence changes were converted to concentrations of thioredoxin oxidized per second using calibration curves, giving a linear relationship with respect to the peroxidase for both the *E. histolytica* (open circles) and *E. dispar* (closed circles) enzymes. (B) Assays were conducted as in (A), but included 0.15 μM of the *E. dispar* peroxiredoxin, 10 μM reduced thioredoxin and 500 μM of peroxide, including: hydrogen peroxide (H_2O_2), ethyl hydroperoxide (EHP), *tert*-butyl hydroperoxide (*t*-BHP) or cumene hydroperoxide (CHP). (C) Assays were conducted as in (B), except that 0.115 μM of the *E. dispar* enzyme was used, the hydrogen peroxide concentration was varied from 7 to 1000 μM, and data were fit to the Michaelis–Menten equation (solid line).

peroxiredoxins (reviewed in [2,31,33]. In early studies, a "thiol-specific antioxidant" (TSA) was identified in *Saccharomyces cerevisiae* [34], as was an alkyl hydroperoxide (AhpC) reductase system, which is regulated by the hydrogen peroxide-sensitive transcriptional activator, OxyR, in

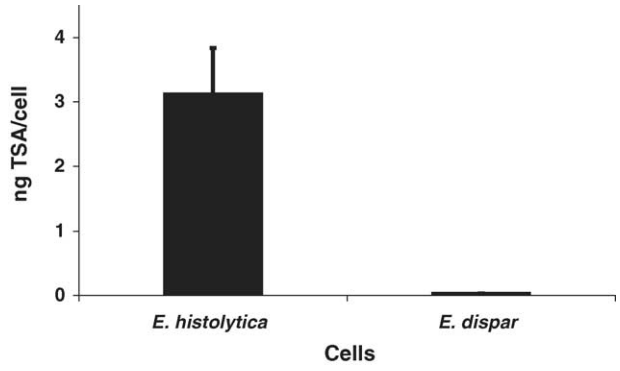

Fig. 5. The amount of peroxiredoxin in *E. histolytica* and *E. dispar* measured by capture ELISA. Plates were coated with monoclonal antibodies FP16 and FP21 (250 ng each) and cell lysates (350–9200 ng protein) added. Binding was detected with HRP labeled FP10 and SureBlue Researve (KPL) and the amount of peroxiredoxin determined from a standard curve of recombinant proteins. Data represent the mean and standard error of four determinations.

Salmonella typhimurium [35]. The peroxiredoxin genes are well conserved, and their protein products are abundant, suggesting a fundamental and essential function of peroxiredoxins in the defense against oxidative stress in living organisms (reviewed in [31,33]).

Although *E. histolytica* and *E. dispar* are morphologically identical, they are genetically distinct species [1], and only *E. histolytica* is capable of invasion. During invasion, *E. histolytica* trophozoites must attach to the intestinal mucosa, invade the epithelium and resist immune and non-immune host defenses, including H_2O_2 and reactive oxygen species generated by host immune cells. Therefore, the peroxiredoxin of *E. histolytica* is likely to play a key role in protection from oxidant stress during invasion.

We found that recent clinical isolates of *E. dispar* are more susceptible to killing by peroxides in vitro than *E. histolytica* (Fig. 1). This supports the earlier findings of Ghadirian et al. [10] who found an attenuated strain of *E. histolytica* was more susceptible to H_2O_2 than a strain, which could cause abscesses in animal models. We chose to test the differences in peroxide killing in a more physiologic system, using recent clinical isolates [36]. Similar significant differences in peroxide killing could not be demonstrated with axenic *E. histolytica* or monoxenic *E. dispar* because the isolates could not be maintained long-term in identical culture media.

To further characterize the *E. histolytica* and *E. dispar* peroxiredoxins, we cloned both genes for recombinant protein expression. The resulting 732 bp gene of clinical *E. dispar* isolate is compared to the *E. histolytica* gene and a previously published sequence from *E. dispar* [17] (Fig. 3). Both the *E. histolytica* and *E. dispar* enzymes have two active site cysteines, Cys47 and Cys170 (*S. cervesiae* numbering), showing that they belong to the 2-cys group of peroxiredoxins (reviewed in [31,33]). The 1-cys peroxiredoxins lack the conserved Cys170 residue, and are thought to be more primitive than 2-cys groups. The disulfide bonds formed during reaction with hydroperoxides can be regenerated with small chemical thiols such as DTT, or physiologically with reduced thioredoxins or species-specific thioredoxin or glutaredoxin

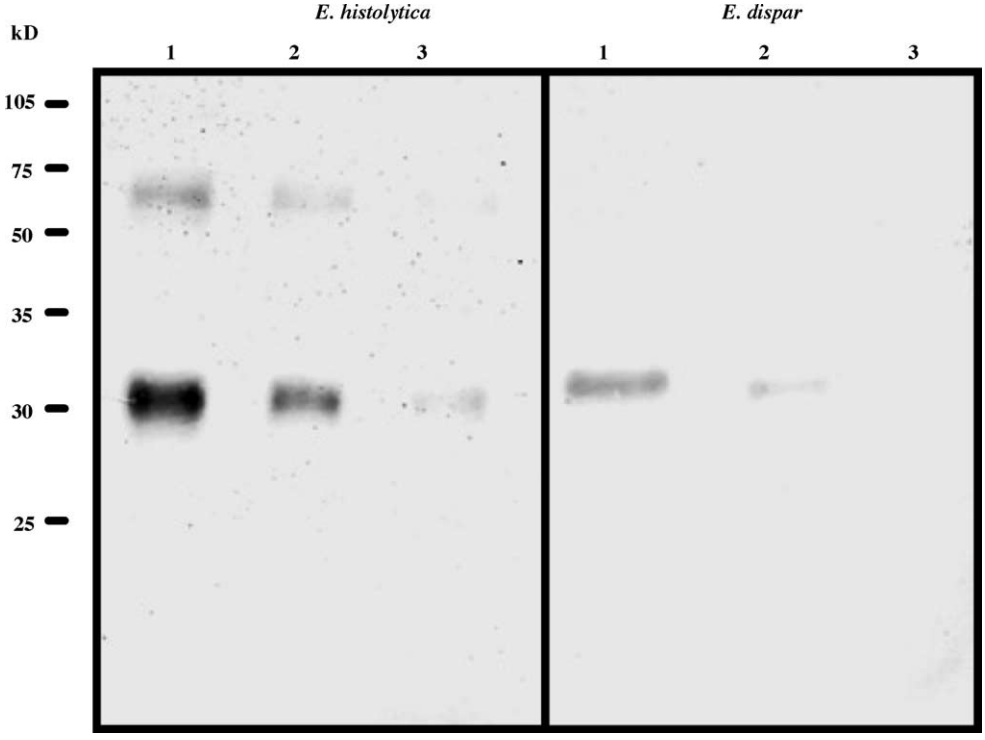

Fig. 6. Immunoblots of *E. histolytica* and *E. dispar* lysates. Lysates (1.125–4.6 μg protein) were electrophoresed, transferred to nitrocellulose membranes and detected with mouse monospecific antibody. Lane 1, 4.6 μg of protein; lane 2, 2.3; lane 3, 1.15.

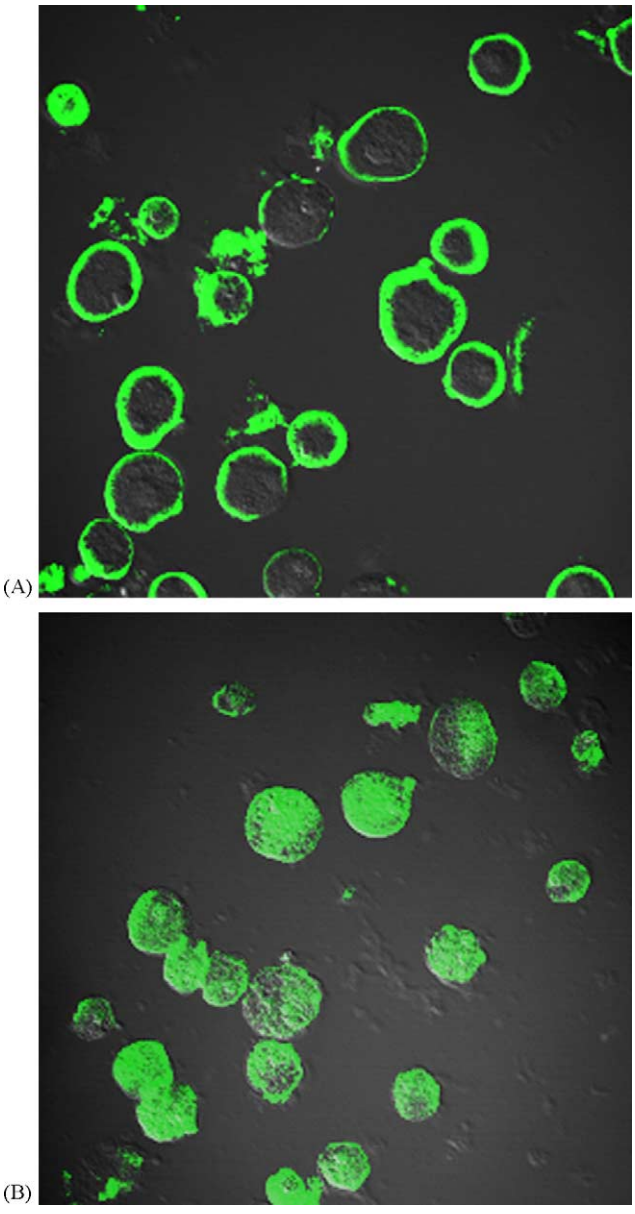

Fig. 7. The localization of peroxiredoxin of axenic *E. histolytica* (A) and *E. dispar* (B) by confocal microscopy. Trophozoites were fixed in 10% formalin and permeabilized with 0.1% Triton X-100. They were then incubated with 5 μg/ml of the monoclonal antibody FP10, and reacted with Alexa 488-conjugated goat anti-mouse antibody (1:200 dilution). Cells were imaged with a Zeiss 510 laser scanning confocal microscope with an argon/krypton laser. Negative controls using isotype matched antibodies and the conjugates alone revealed no specific staining (not shown).

homologues. In most bacteria, a specialized protein, AhpF, comprises both a flavoprotein disulfide reductase segment similar to thioredoxin reductase and an AhpC-interacting N-terminal domain related to thioredoxin [30]. The candidate regenerating protein(s) have not been definitely identified in *E. histolytica*. Rat brain thioredoxin/thioredoxin reductase, but not bacterial AhpF, could reactivate the amebic peroxiredoxin in vitro [6]. Trypanothione, a conjugate of exogenous glutathione and spermidine, is the major regenerating thiol compound in trypanosomes [37] and leishmania [38]. There are conflicting reports whether trypanothione exists in *Entamoeba* [39–40], but Ondarza's group reports the recent cloning of a trypanothione reductase gene in *E. histolytica* (R. Ondarza, personal communication).

Wood et al. have recently identified structural features of higher eukaryotic 2-cysteine peroxiredoxins: a GGLG motif and an additional C-terminal extension [7]. These motifs confer sensitivity to overoxidation allowing peroxiredoxins to act as "floodgates" enabling signal transduction at higher H_2O_2 levels [7]. These sequences are absent in *Entamoeba* and some other protozoan parasites such as kinetoplastids and *Giardia*, suggesting they may be an evolutionary intermediate to the dual function peroxiredoxin.

To compare the activity of the individual enzymes from *E. histolytica* and *E. dispar*, the active recombinant enzymes were expressed and purified (Fig. 3). Bruchhaus et al. [9] had shown that the cysteine-rich 40 amino acids of N-terminal region was important for activity, and our constructs containing the protein as a thioredoxin fusion protein were also enzymatically active. There were no significant differences between the activities of the two enzymes toward hydrogen peroxide, cumene hydroperoxide, *t*-butyl hydroperoxide or ethyl hydroperoxide (Fig. 4). These results are not surprising considering the conservation of the two active-site cysteinyl residues and the otherwise high degree of homology in amino acid sequence between the two peroxiredoxins. Interestingly, the recombinant peroxiredoxin of the free-living amebae, *E. moshkovskii*, had equivalent to slightly greater activity than the *E. histolytica* enzyme and more protective activity against oxidative-nicking of supercoiled plasmid DNA [18]. Although it would have been ideal to measure the thioredoxin-dependent peroxidase activity in cell lysates directly, the activity depends on the amounts of both peroxiredoxin and thioredoxin (equivalent) proteins present in the assay, both of which would be unknown in extracts.

The hypothesis that *E. histolytica* trophozoites would have more peroxiredoxin than *E. dispar* proved true. Both axenic ($p < 0.001$) and clinical isolates (data not shown) of *E. histolytica* had significantly greater amounts of peroxiredoxin than *E. dispar* (Fig. 5), whether corrected to cell number or protein concentration. These results were also confirmed by immunoblots (Fig. 6). *E. moshkovskii* was found to have similar levels of peroxiredoxin mRNA by RT-PCR, but it is a free-living amebae and is exposed to an aerobic environment [18].

E. histolytica and *E. dispar* contain the same classes of genes encoding putative virulence factors, including the cysteine proteinases [41,42], galactose-inhibitable lectin [43] and amebapores [44]. Although these two amebae express peroxiredoxins with more than 90% peptide identity [17], we found that *E. histolytica* expresses significantly more enzyme than *E. dispar*. The peroxiredoxin of *E. histolytica* is likely important in protection from the high-oxygen environment of the human host and from oxidative attack by activated host phagocytic cells during amebic invasion, while the *E. dis-*

par enzyme may have a major role in protection against its own metabolically produced H_2O_2 [17]. Indeed, the peroxiredoxin was recently shown to be upregulated during exposure of trophozoites to a high-oxygen environment [45 and B. Torian, unpublished data].

The cellular localization of the peroxiredoxin of *E. histolytica* has been an area of debate. We found the antigen to be membrane associated by radioiodine and indirect fluorescent antibody labeling of live parasites, immunoelectron microscopy [12,24], and now confocal microscopy (Fig. 6). Flores et al. [8] demonstrated that the 29-kDa protein forms a 60-kDa dimer and a high-molecular-mass oligomer on the cell surface through disulfide bonds, and is the major free thiol-containing surface protein of *E. histolytica*. However, cytoplasmic localization was reported by other groups using indirect fluorescent antibodies [46] and immunoblots of soluble versus membrane proteins [9]. There is no structural basis to suggest that the peroxiredoxin is a transmembrane protein, but it is surface associated. Surface localization of peroxiredoxins has now been suggested in other organisms, including *Bacillus anthracis* [47] and *Cryptococcus neoformans* [48] (J. Lodge, personal communication), and the eggs of *Schistosoma mansoni* [49]. The fact that the peroxiredoxin in Leishmania acts as a vaccine is further evidence for surface location in that organism [21].

One explanation for the membrane association of the peroxiredoxin of *E. histolytica* may be the recent finding that it binds to the cytoplasmic domain of the *N*-acetylgalactosamine inhibitable (GalNAc) lectin, the major surface lectin of *E. histolytica* mediating adherence to host cells [50]. Moreover, the peroxiredoxin and the lectin co-localized when amebae were incubated with neutrophils or $CaCo_2$ polarized epithelial monolayer, as assessed by co-immunoaffinity purification and confocal microscopy. This suggests that the peroxiredoxin might localize to the site of parasite:host cell contact where it can effectively counteract oxidants generated by host cells, and thus facilitate invasion of the host. The explanation for the lack of binding of the *E. dispar* peroxiredoxin to its lectin remains to be determined but might be attributed to differences in amino acids at the 3′ end of the *E. dispar* peroxiredoxin.

Thus, our findings help explain how *E. histolytica* trophozoites, which are exposed to oxidants in the aerobic host environment, reactive oxygen species generated by immune cells, and products of their own antioxidant enzymes, Fe-SOD and flavin reductase, are able to survive and cause invasive disease. Both *E. histolytica* and *E. dispar* have homologous genes encoding peroxiredoxins (90.5% amino acid identity [17]), but quantitatively, there are significant differences. *E. histolytica* has a surface-localized peroxiredoxin, which may aid in the transition from anaerobic to aerobic environment in the human host, whereas *E. dispar* never leaves the anaerobic bowel. Both axenic and clinical strains of *E. histolytica* are more resistant to H_2O_2 and possess quantitatively more peroxiredoxin per trophozoite. Therefore, we have shown that the peroxiredoxin is likely a virulence factor for *E. histolytica*, which can protect the trophozoite from the oxidant attack of activated host cells, and thereby facilitate the invasion of the host. Indeed, the recombinant *E. histolytica* 29-kDa antigen was partially protective in the gerbil model of amebic liver abscesses [20]. Peroxiredoxins are important in the host–parasite interaction, and may be future candidate targets for antiamebic drugs and vaccines.

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