Transcriptional profile of the homologous recombination machinery and characterization of the EhRAD51 recombinase in response to DNA damage in *Entamoeba histolytica*

Mavil López-Casamichana¹, Esther Orozco², Laurence A Marchat³ and César López-Camarillo*¹

Address: ¹Posgrado en Ciencias Genómicas, Universidad Autónoma de la Ciudad de México, México DF, México, ²Departamento de Patología Experimental, CINVESTAV-IPN, México DF, México and ³Programa Institucional de Biomedicina Molecular, ENMH-IPN, México DF, México

Email: Mavil López-Casamichana - gerry2410@yahoo.com; Esther Orozco - esther@cinvestav.mx; Laurence A Marchat - lmarchat@gmail.com; César López-Camarillo* - genomicas@yahoo.com.mx

* Corresponding author

Abstract

Background: In eukaryotic and prokaryotic cells, homologous recombination is an accurate mechanism to generate genetic diversity, and it is also used to repair DNA double strand-breaks. RAD52 epistasis group genes involved in recombinational DNA repair, including mre11, rad50, nsb1/xrs2, rad51, rad51c/rad57, rad51b/rad55, rad51d, xrc2, xrc3, rad52, rad54, rad54b/rdh54 and rad59 genes, have been studied in human and yeast cells. Notably, the RAD51 recombinase catalyses strand transfer between a broken DNA and its undamaged homologous strand, to allow damaged region repair. In protozoan parasites, homologous recombination generating antigenic variation and genomic rearrangements is responsible for virulence variation and drug resistance. However, in *Entamoeba histolytica* the protozoan parasite responsible for human amoebiasis, DNA repair and homologous recombination mechanisms are still unknown.

Results: In this paper, we initiated the study of the mechanism for DNA repair by homologous recombination in the primitive eukaryote *E. histolytica* using UV-C (150 J/m²) irradiated trophozoites. DNA double strand-breaks were evidenced in irradiated cells by TUNEL and comet assays and evaluation of the EhH2AX histone phosphorylation status. In *E. histolytica* genome, we identified genes homologous to yeast and human RAD52 epistasis group genes involved in DNA double strand-breaks repair by homologous recombination. Interestingly, the *E. histolytica* RAD52 epistasis group related genes were differentially expressed before and after UV-C treatment. Next, we focused on the characterization of the putative recombinase EhRAD51, which conserves the typical architecture of RECA/RAD51 proteins. Specific antibodies immunodetected EhRAD51 protein in both nuclear and cytoplasmic compartments. Moreover, after DNA damage, EhRAD51 was located as typical nuclear foci-like structures in *E. histolytica* trophozoites. Purified recombinant EhRAD51 exhibited DNA binding and pairing activities and exchanging reactions between homologous strands in vitro.

Conclusion: *E. histolytica* genome contains most of the RAD52 epistasis group related genes, which were differentially expressed when DNA double strand-breaks were induced by UV-C irradiation. In response to DNA damage, EhRAD51 protein is overexpressed and relocalized in nuclear foci-like structures. Functional assays confirmed that EhRAD51 is a bonafide recombinase. These data provided the first insights about the potential roles of the *E. histolytica* RAD52 epistasis group genes and EhRAD51 protein function in DNA damage response of this ancient eukaryotic parasite.
Background

Entamoeba histolytica, the protozoan causative of human amoebiasis, has a world-wide distribution with a higher prevalence in developing countries, affecting more than 50 million people each year [1]. Trophozoites show a dramatic virulence variability that could be related to great genome plasticity [2]. Frequent ploidy changes, unscheduled gene amplification and duplication have been reported [3,4], and it has been largely assumed that these processes are linked to genetic rearrangements, although no direct experimental evidence has been provided yet.

In eukaryotic and prokaryotic cells, homologous recombination (HR) is an accurate mechanism to generate genetic diversity. HR is also used by cells to properly repair the DNA double strand-breaks (DSBs). Generally, this kind of damage is produced by genotoxic agents or during cellular processes like meiotic division, telomere maintenance, and restoration of collapsed replication forks in the course of DNA synthesis [5-7]. Cellular response to DNA DSBs activates a complex network of proteins that transiently arrests cell cycle and enhances DNA repair mechanisms. Particularly, Saccharomyces cerevisiae H2A and Homo sapiens H2AX histones are rapidly phosphorylated in the chromatin micro-environment surrounding DNA DSBs, inducing nucleosome remodeling to promote accumulation of checkpoint and DNA repair proteins at these sites [8]. In case of extreme DNA damage, cells are targeted to apoptosis [9]. Additionally, HR is also a useful tool to analyze gene function by gene targeting and gene knock out approaches [10].

Molecular genetics of HR DNA repair has been well preserved throughout evolution. RAD52 epistasis group genes involved in DNA DSB repair, including mre11, rad50, nsb1/xxs2, rad51, rad51c/rad57, rad51b/rad55, rad51d, xrc2, xrc3, rad52, rad54, rad54b/rdh54 and rad59 genes, have been identified in human and yeast cells [11]. Pivotal protein in HR pathway is the RAD51 recombinase, which catalyses strand transfer between a broken DNA and its undamaged homologous strand, allowing damaged region to be repaired [12]. Strand exchange reaction is initiated by RAD51-coating of single-stranded DNA (ssDNA) released from DSBs, to generate a nucleoprotein filament. This active thread binds the intact double-stranded DNA (dsDNA) substrate, searching and locating homologous sequences, and promoting DNA strand exchange in an ATP-dependent manner, forming a heteroduplex structure called D-loop [13]. After DNA damage, RAD51 protein has been observed in nuclear complexes forming discrete foci, which are considered as the recombinational DNA repair sites [14].

HR remains the predominant mechanism to repair DSBs in lower eukaryotes [15], RAD51 proteins have been identified in Trypanosoma brucei and Plasmodium falciparum parasites, which perform HR to switch the expression of genes encoding surface membrane glycoproteins and generate antigenic variation [16-18]. Furthermore, recombinational rearrangements are responsible for amplification of the multidrug resistance pfmdr1 gene in P. falciparum [19], demonstrating the relevance of HR to generate genomic versatility and plasticity in protozoan parasites.

In this paper, we identified and analyzed the mRNA expression profile of E. histolytica RAD51 epistasis group related genes in response to DNA damage. Additionally, we presented experimental evidence of EhRAD51 function as a recombinase, which suggest its potential role in DNA damage response. These findings constitute the initial efforts to understand the DNA repair mechanism in E. histolytica that will contribute to the further elucidation of events regulating genome integrity and variability in this early-branch protozoan.

Results

High dose of UV-C light induces DNA fragmentation in trophozoites

It has been shown in a wide variety of cells that X-rays exposure, UV irradiation and chemicals activate cellular responses to DNA repair [20]. To initiate the study of the mechanisms involved in DNA repair in E. histolytica, we used UV-C light irradiation to induce DNA damage in trophozoites. Our experiments showed that during the first 12 h after irradiation with 254 nm UV-C (150 J/m²), cell survival was not significantly affected (Fig. 1A). Using the same experimental conditions, we analyzed the presence of 3′-hydroxyl DNA ends by TUNEL and FACS assays. In untreated trophozoites, FACS analysis evidenced the presence of <1% TUNEL positive cells; meanwhile, 30 min after treatment, 57.4 ± 2.74% of UV-C irradiated cells showed DNA fragmentation (Fig. 1B, upper panels). DNA damage reduction was observed at 3, 6 and 12 h after treatment (27.11 ± 4.84, 8.79 ± 3.36 and 0.77 ± 2.59%, respectively). Propidium iodide stained cells were checked under the fluorescence microscope to confirm the absence of cytoplasmic stain (Fig. 1B, lower panels).

The comet assay (single-cell gel electrophoresis) is widely used to measure DNA damage and repair. Results obtained through neutral comet assay (Fig. 1C) confirmed the induction of DSBs in trophozoites by UV-C treatment. Typical comet-like structures were observed at 30 min and 3 h, while a reduction of the DNA tails was observed at 6 h after UV-C treatment. As expected, 12 h after the genotoxic insult, DNA migration was similar to the control untreated cells (No UV-C). Taking altogether, these data indicated that UV-C irradiation efficiently induced DNA damage and consequently, repair mechanisms were activated to restore DNA integrity allowing cell survival.
Early EhH2AX histone phosphorylation correlates with the presence of DNA DSBs

DNA DSBs induce early phosphorylation of yeast H2A (major H2A closer to mammalian H2AX) and human H2AX histones on a conserved serine residue located in the SQ motif at C terminus, producing \( \gamma \)H2A and \( \gamma \)H2AX, respectively [21]. As in yeast, *E. histolytica* seems to have replaced the canonical H2A with H2AX [22]. Two genes (locus EHI_126210 and EHI_188960) that encode putative proteins with 55 and 57% identity (e-value 2e-27 and
2e-28) to yeast H2A and human H2AX histones, respectively, were found in the *E. histolytica* genome. These genes predict two 17.6 kDa conserved paralogous H2AX proteins that share 93% identity. Notably, both contain the H2AX exclusive SQ motif with the potentially phosphorylatable serine residue (S156) (Fig. 2A).

Taking advantage of the high conservation between *H. sapiens* and *E. histolytica* H2AX C-terminus, we performed Western blot assays using the anti-human γH2AX antibody to detect serine-phosphorylated EhH2AX homologues (γEhH2AX) in cytoplasmic (CE) and nuclear (NE) extracts of trophozoites. Protein amount and integrity were confirmed on Coomassie blue stained-gels (data not shown). In NE from non-irradiated cells, we identified a 17-kDa weak band, which corresponds to the expected molecular weight of γEhH2AX histones (Fig. 2B, lane 2). Interestingly, 10 min after UV-C irradiation, this band was five-fold more intense, suggesting an increase in the amount of nuclear γEhH2AX, and 30 min after treatment no band was found (Fig. 2B, lanes 4 and 6). However, these assays did not allow us to distinguish whether one or both EhH2AX proteins were phosphorylated. In contrast, no signals were observed in CE (Fig. 2B, lanes 1, 3 and 5). We used as an integrity control an anti-EhPAP serum, which recognized the 63-kDa EhPAP protein [23] in non-irradiated and irradiated trophozoites (Fig. 2B, middle panel). In addition, an anti-actin monoclonal antibody, used as control for cell fractionation, strongly detected the expected 42-kDa band in CE and a slight signal in NE, as expected for a major component of cytoskeleton (Fig. 2B, lower panel). These data showed that UV-C irradiation of trophozoites is a useful model to generate DNA DSBs and study DNA repair in *E. histolytica*.

**E. histolytica genome contains RAD52 epistasis group related genes**

In order to investigate the presence of RAD52 epistasis group related genes in *E. histolytica* genome, we surveyed the parasite Pathema database (Table 1). We found *Ehmre11, Ehrad50* and *Ehnbs1* genes, which could encode the *E. histolytica* putative MRE11-RAD50-NBS1 protein complex that functions as the primary sensor of DNA DSBs in other organisms [9]. Both *EhMRE11* and *EhRAD50* proteins exhibited 32 to 23% identities (e-values from 3e-49 to 9e-36) with *S. cerevisiae* and *H. sapiens* orthologous proteins, respectively; whereas the *EhNBS1* sequence appears to be more divergent (17 to 24% identity and e-values from 0.003 to 0.002). *E. histolytica* also contains genes encoding the putative recombinase EhRAD51 and its paralogous protein EhRAD51C. EhRAD52, EhRAD54, EhRAD54B and EhRAD59 (EhRAD52/22 in Pathema database) predicted proteins are also encoded in the *E. histolytica* genome. As in yeast,

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**Figure 2**

Identification and immunodetection of phosphorylated EhH2AX histones (γEhH2AX). **A.** Multiple sequence alignments of *E. histolytica* and *H. sapiens* H2AX histones sequences. Black boxes, identical residues; grey boxes, conserved substitutions; open box, C-terminal SQ motif. Arrowhead denotes the potential phosphorylatable serine residue (S156). Numbers at left are relative to the position of the initial methionine in each protein. **B.** Immunodetection of phosphorylated γEhH2AX polypeptides by Western blot assays using anti-human γH2AX polyclonal antibody (upper panel). CE, cytoplasmic extracts; NE nuclear extracts. Lanes 1 and 2, non-irradiated (No UV-C) trophozoites; lanes 3 and 4, irradiated trophozoites (10 min after UV-C treatment); lanes 5 and 6, irradiated (UV-C) trophozoites (30 min after UV-C treatment). Controls using anti-EhPAP and anti-actin antibodies (middle and bottom panels, respectively) are shown.
Table 1: Comparison of E. histolytica, H. sapiens and S. cerevisiae RAD52 epistasis group proteins

| Entamoeba histolytica | Homo sapiens | Saccharomyces cerevisiae |
|-----------------------|-------------|-------------------------|
| **Predicted protein** | **Size (aa)** | **GeneBank ID** | **Locus name a** | **Protein** | **Accession number b** | **Size (aa)** | **e-value** | **H (%)** | **I (%)** | **Protein** | **Accession number b** |
| EhrMRE1I | 596 | XM_651393 | EHI_125910 | MRE11 | P49959 | 708 | 3e-49 | 51 | 32 | MRE11 | P32829 | 692 | 1e-33 | 45 | 26 |
| EhrMRE1I-like | 223 | XM_644963 | EHI_077650 | MRE11 | P49959 | 708 | 3e-23 | 51 | 25 | MRE11 | P32829 | 692 | 1e-20 | 49 | 27 |
| EhrRAD50 | 1241 | XM_647783 | EHI_079960 | RAD50 | Q92878 | 1312 | 2e-44 | 40 | 23 | RAD50 | P12753 | 1312 | 2e-43 | 46 | 27 |
| EhrNBS1 | 764 | XM_647447 | EHI_098770 | NBS1 | Q6IQ31 | 754 | 0e-022 | 40 | 24 | XR52 | P33301 | 854 | e+3 | 28 | 17 |
| EhrRAD51 | 367 | XM_648984 | EHI_031220 | RAD51 | Q6E609 | 339 | e-128 | 83 | 71 | RAD51 | P25454 | 400 | e-24 | 78 | 60 |
| EhrRAD51C | 284 | XM_619126 | EHI_122860 | RAD51C | Q43502 | 376 | 3e-17 | 51 | 28 | RAD57 | P32829 | 460 | e-109 | 47 | 27 |

**Note:**
- a: E. histolytica Pathema database
- b: Swiss-Prot/TrEMBL databases

Rad51 paralogs (rad51b, rad51d, xrc2 and xrc3) that participate in HR in vertebrates were not found in E. histolytica (Table 1). In conclusion, E. histolytica genome contains a conserved set of repair genes, which suggests that it is skilled to perform recombinational DNA repair.

**E. histolytica genes of the RAD52 epistasis group are differentially expressed in response to UV-C irradiation**

As a first step towards establishing the role of the E. histolytica RAD52 epistasis group related genes, we evaluated their mRNA expression by semi-quantitative RT-PCR using the UV-C irradiation model described above. Most genes exhibited a differential mRNA expression profile before and after irradiation (Fig. 3). EhrMRE1I, EhrRAD51, EhrRAD51c and EhrRAD52 genes were transcribed at a very low level in non-irradiated trophozoites; meanwhile mRNA expression was induced from 30 min to 12 h after genotoxic damage. Particularly, the EhrRAD51 N-terminus has stacking motif or ATP cap (342–350 aa residues) at the C-terminus, which are essential for nucleofilament assembly and ATP hydrolysis in RAD51/RECA-like recombinases, respectively (Additional file 1).

The predicted EhrRAD51 conserves the typical architecture of RECA/RAD51 family members

Since RAD51 recombinases are considered as key enzymes in HR and DNA repair processes in many organisms [24], we focused on the characterization of the E. histolytica EhrRAD51 protein. Ehrad51 is an intron-less 1101 bp gene, which encodes a 367 amino acids (aa) polypeptide (40.3-kDa). Sequence similarity searches by BLAST showed the lowest e-values (from 3e-29 to 2e-20) and high identity (from 59 to 75%) with many eukaryotic RAD51 proteins, from plants to human, including protozoan parasites. Moreover, EhrRAD51 showed 51% and 36% identity with Methanococcus voltae RADA and Escherichia coli RECA bacterial recombinases, respectively (Additional file 1).

Amino acid sequence alignment of EhrRAD51 protein with yeast and human RAD51 orthologs revealed that these proteins share functional and structural conserved motifs (Fig. 4A). EhrRAD51 contains the putative polymerization motif (110–113 aa residues), which tethers individual subunits to form quaternary assemblies in human RAD51 protein [24] (Additional file 2). We also identified the ATPase Walker A or phosphate binding loop (P-loop; 152–159 aa residues) and Walker B motifs (240–249 aa residues), the ssDNA binding loops L1 (255–264 aa residues) and L2 (293–311 aa residues), as well as the ATP-stacking motif or ATP cap (342–350 aa residues) at the C-terminus, which are essential for nucleofilament assembly and ATP hydrolysis in RAD51/RECA-like recombinases [26,27]. Remarkably, the EhrRAD51 N-terminus has a low-complexity region of 34-aa highly enriched in glutamic residues, which is not present in homologous...
proteins (Fig. 4A). Phylogenetic relationships among EhRAD51 and RAD51/RECA related proteins from diverse organisms, revealed a progressive evolution from eubacteria to eukaryotes, being EhRAD51 more related to protozoan recombinases (Fig. 4B).

The EhRAD51 protein is overexpressed in response to DNA damage
The recombinant EhRAD51 protein (rEhRAD51) was expressed in E. coli BL21 (DE3) plysS strain as a 6x His-tagged fusion polypeptide and subsequently purified by affinity chromatography (Fig. 5A, lanes 3 and 4). By Western blot assays using monoclonal anti-6xHis tag antibodies, the purified rEhRAD51 was detected as a single 47 kDa band, which was slightly higher than the 44.1 kDa expected weight (Fig. 5B, lane 2). Then, rEhRAD51 was used to generate rabbit polyclonal anti-EhRAD51 antibodies. These antibodies recognized the 47 kDa rEhRAD51 band (Fig. 5B, lane 4), whereas the preimmune serum, used as negative control, did not detect any signal (Fig. 5B, lane 3). To evaluate the expression of the native EhRAD51 in E. histolytica, we performed Western blot assays using anti-EhRAD51 antibodies and protein extracts from irradiated and non-irradiated trophozoites. Antibodies reacted with a weak 46 kDa band in CE from non treated trophozoites, but not signal was detected in NE (Fig. 5C, higher panel, lanes 1 and 2). Meanwhile, at 30 min after UV-C irradiation, antibodies strongly detected the expected 41 kDa endogenous EhRAD51 in CE, but not in NE (Fig. 5C, higher panel lanes 3 and 4). Intriguingly, antibodies also detected a 46 kDa band in both NE and CE from UV-C irradiated trophozoites, which may correspond to a modified form of the 41 kDa protein. The specificity of anti-EhRAD51 antibodies was confirmed performing a similar Western blot assay using anti-EhRAD51 antibodies previously pre-incubated with...
purified rEhRAD51 protein and the recognition of both 46 and 41 kDa proteins was specifically inhibited (data not shown). In addition, the use of anti-EhPAP and anti-actin antibodies confirmed protein integrity and cell fractionation of CE and NE (Fig. 5C, middle and lower panels). Our findings showed that EhRAD51 was overexpressed in response to UV-C irradiation, and distributed in both nuclear and cytoplasmic compartments.

### EhRAD51 relocalizes into nuclear foci-like structures in response to DNA damage

In order to better characterize the EhRAD51 expression and function, we investigated its subcellular location in trophozoites through immunofluorescence and laser confocal microscopy. In agreement with the Western blot results, EhRAD51 was detected at low levels in the cytosol of non-irradiated trophozoites (Fig. 6, panels A-D), whereas at 30 min after UV-C irradiation we noted a dramatic accumulation of cytoplasmic EhRAD51 protein. Interestingly, we also observed a scattered distribution of EhRAD51 typical foci-like structures in the nucleus (Fig. 6, panels E-H).

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**Figure 4**

**Comparison of the predicted amino acids sequence of EhRAD51 with other RAD51 proteins.**

A. Multiple sequence alignments of functional domains of RAD51 from *E. histolytica* (EhRAD51), *S. cerevisiae* (ScRAD51) and *H. sapiens* (HsRAD51) proteins. Upper panel: Glutamate-rich region, polymerization motif (PM), Walker A and B motif, L1 and L2 regions and ATP cap appear as colored boxes. Lower panel: black boxes, identical aa; grey boxes, conserved substitutions; open box, Glutamate-rich region. Numbers at the left are relative to the position of the initial methionine in each protein. Accession numbers and protein lengths are indicated in the Supplementary Table S1.

B. Phylogenetic relationships between EhRAD51 and RECA/RAD51 family members. The unrooted tree was created with the MEGA 3.1 program using the Neighbor Joining algorithm based on ClustalW alignments of complete amino acids sequences. Numbers above the tree nodes indicate the percentage of times that the branch was recovered in 1000 replications.
panels E-H). Three hours later, the cytoplasmic signal diminished, while nuclear foci-like structures remained (Fig. 6, panels I-L). At 12 h after genotoxic damage, both cytoplasmic and nuclear EhRAD51 signals were very weak, being EhRAD51 foci-like structures scarce (Fig. 6, panels M-P). Quantification of nuclear foci like-structures by statistical microscopic analysis showed that about 60% of the cells contained at least one focus at 30 min after UV-C irradiation (Fig. 6Q). These findings confirmed that EhRAD51 was up-regulated after UV-C irradiation and suggested that it was redistributed into the nucleus during the first 3 h after DNA damage.

**rEhRAD51 exhibits DNA binding activity in vitro**

*In silico* analysis of the EhRAD51 aa sequence evidenced the presence of two putative DNA binding domains. To verify that EhRAD51 is a DNA binding protein, we performed EMSA using increasing amounts of purified rEhRAD51 protein and a fixed concentration of radiolabeled 50-bp ssDNA or 270-bp dsDNA fragments as probes. In order to discard interactions of contaminant *E. coli* proteins with DNA probes, we used mock purified fractions obtained from untransformed bacteria as a negative control. Results showed that incubation of rEhRAD51 with ssDNA and dsDNA probes resulted in five DNA-protein complexes (C1-C5) formation, suggesting that alternative populations of RAD51 protomers were associated to each DNA probes (Fig. 7A and 7B, lanes 2 to 4). The fastest migration ssDNA-protein complex C1 that was also formed with the mock fraction was considered as unspecific (Fig. 7A, lanes 5 to 7). No complexes were formed in the EMSA control performed with the dsDNA probe (Fig. 7B, lanes 5 to 7). Notably, the abundance of slow migration DNA-protein complexes appeared to increase in the presence of the highest rEhRAD51 amount (Fig. 7A and 7B, lanes 2 to 4). These results showed that rEhRAD51 was able to efficiently bind both ssDNA and dsDNA substrates in vitro.

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**Figure 5**

**Expression and immunodetection of EhRAD51.**

**A.** Expression and purification of rEhRAD51-6x His-tagged protein. Bacterial proteins were separated through 10% SDS-PAGE and gels were stained with Coomassie blue. Lane 1, molecular weight markers; lane 2, non-induced bacterial extract (30 μg); lane 3, IPTG-induced bacterial extract (30 μg) before passing through the Ni2+-NTA affinity column; lane 4, affinity purified polypeptide from IPTG-induced bacteria extract. Arrowhead, 47-kDa rEhRAD51. **B.** Immunodetection of rEhRAD51 polypeptide. Western blot assays were performed using non-induced bacterial extract (lane 1) and purified rEhRAD51 (lanes 2 to 4). Lanes 1 and 2; anti-6x His tag antibodies; lane 3, preimmune serum; lane 4, specific rabbit antibodies raised against rEhRAD51. Arrowhead, 47-kDa rEhRAD51. **C.** Immunodetection of *E. histolytica* endogenous EhRAD51 by Western blot assays using specific anti-EhRAD51 antibodies. CE, cytoplasmic extracts; NE nuclear extracts. Lanes 1 and 2, non-irradiated (No UV-C) trophozoites; lanes 3 and 4, irradiated (UV-C) trophozoites (30 min after UV-C treatment). Upper panel: arrowhead, 41-kDa EhRAD51; asterisk, 46-kDa EhRAD51. Controls using anti-EhPAP and anti-actin antibodies (middle and bottom panels, respectively) are shown.
**rEhRAD51 exhibits homologous DNA strand transfer activity in vitro**

In order to evaluate the homologous DNA strand transfer function of the rEhRAD51 protein, we performed a pairing assay involving the D-loop structure formation as described in Experimental procedures. Results revealed that rEhRAD51 was able to shift the electrophoretic mobility of the radioactive-labeled 200-bp ssDNA probe incubated with homologous circular dsDNA (Fig. 7C, lanes 2 to 4). This indicated that rEhRAD51 was able to catalyze ssDNA transfer to homologous dsDNA forming the three-stranded pairing molecule or D-loop structure, which has a reduced electrophoretic mobility in comparison with the ssDNA probe. The D-loop formation specificity was confirmed by incubation of rEhRAD51 and ssDNA probe in the absence of homologous dsDNA substrate (Fig. 7C, lane 5), and in the presence of a heterologous dsDNA substrate (Fig. 7C, lane 6), since no complex was observed. In addition, we did not observe any D-loop structure in the absence of rEhRAD51 (Fig. 7C, lane 1). Densitometric analysis of radioactive products showed that D-loop structure formation using 7.5 μg of rEhRAD51 was 3.6 and 1.7-fold higher than with 2.5 and 5 μg of rEhRAD51, respectively (Fig. 7D). These results indicated that EhRAD51 protein was able to catalyze specific DNA paring and exchange between DNA homologous strands in vitro.

**Discussion**

While non-homologous end joining plays a major role in DSB DNA repair in higher eukaryotes including mammals, HR remains the predominant mechanism to repair...
Figure 7
DNA-binding and homologous strand transfer activities of rEhRAD51. A. Partially-purified rEhRAD51 was incubated with $[\gamma^{32}P]$dATP labeled ssDNA and interactions were resolved through non-denaturing PAGE. Lane 1, free probe. Lanes 2 to 4, ssDNA incubated with increasing amounts of rEhRAD51 (2.5, 5 and 7.5 μg, respectively); lanes 5 to 7, ssDNA incubated with increasing concentrations of mock purified fraction (2.5, 5 and 7.5 μg) as control. Protein-DNA complexes ($C_1$ to $C_7$) are shown by arrowheads.

B. Partially purified rEhRAD51 was incubated with $[\alpha^{32}P]$dATP labeled dsDNA and interactions were resolved through PAGE. Lane 1, free probe. Lanes 2 to 4, dsDNA incubated with increasing amounts of rEhRAD51 (2.5, 5 and 7.5 μg, respectively); lanes 5 to 7, dsDNA incubated with increasing concentrations of mock purified fraction E. coli elution fraction (2.5, 5 and 7.5 μg) as control. Protein-DNA complexes ($C_1$ to $C_7$) are shown by arrowheads. C. D-loop reactions containing 10,000 cpm of $[\gamma^{32}P]$dATP-labeled oligonucleotide, circular dsDNA and 0, 2.5, 5 and 7.5 μg of partially-purified rEhRAD51 (lanes 1 to 4) were incubated at 37°C for 30 min with 2 mM of ATP. Negative controls were performed without homologous dsDNA (lane 5) and with heterologous dsDNA oligonucleotide instead of homologous dsDNA (lane 6), both of them using 7.5 μg of EhRAD51 elution fraction. Reaction products were analyzed by agarose gel electrophoresis, transferred to nylon membranes and visualized through a Phosphor Imager. D. Densitometric analysis of D-loop products obtained in C. Results are representative of two independent experiments.
this kind of lesions in lower eukaryotes [15]. The high amount of repetitive DNA in protozoan parasites, such as *E. histolytica*, suggests that the genome of these organisms can be potentially recombinogenic. Therefore, the study of HR process in *E. histolytica* may advance our understanding about trophozoites genetic and virulence variability, as well as DNA repair mechanisms.

Here, we developed a 254 nm UV-C light irradiation model, which induces DNA damage in *E. histolytica* trophozoites and activates recombinational DNA repair pathway. Irradiation dose (150 J/m²) and time (8 s) were determined as no lethal conditions for cells in comparison with other UV doses previously evaluated. Growth curves were performed up to 18 h, the doubling time of trophozoites, without observing any significant changes (data not shown). Early phosphorylation of *E. histolytica* H2AX histones after UV-C irradiation was consistent with DNA DSBs formation, suggesting chromatin remodeling and recruitment of histone-phosphorylating enzymes, as observed in other eukaryotic systems [22]. Moreover, *E histolytica* trophozoites survival throughout almost 12 h after irradiation indicated the existence and activation of DNA repair mechanisms. In silico analysis of the *E. histolytica* genome sequence revealed that this pathogen has genes that encode putative EhRAD52 epistasis group members, which participate in recombinational DNA repair in other organisms. Given the place of this ancient protista in the eukaryote phylogenetic scale, EhRAD52 epistasis group had equivalent similarity with homologous proteins from different organisms, such as mammals, plants and other protozoan parasites.

RT-PCR assays evidenced a differential mRNA expression of *E. histolytica rad52* epistasis group genes, before and after DNA damage. Some genes (*Ehnbs1, Ehrad54* and *Ehrad52/22*) were down-regulated after DNA damage, others (*Ehmre11, Ehrad51, Ehrad51-C and Ehrad52*) were up-regulated at different times following genotoxic stimulus, whereas *Ehrad50* mRNA levels were regulated in a variable manner, suggesting a complex transcriptional response. Interestingly, *Ehrad54b* gene did not seem to be transcribed under our experimental conditions. However, in yeast and human, both RAD54 and RAD54B are DNA helicases which participate in the formation of heteroduplex DNA in recombination processes [11]. It is possible that the expression of *Ehrad54* homolog is sufficient to cover this activity in trophozoites, although additional experiments are required to confirm this hypothesis. The absence of a coordinated transcriptional activation of *Ehrad52* epistasis group genes suggest that trophozoites have enough stationary levels of enzymes for DBB repair and the main regulation could be occurring at translational and/or posttranslational level. A further evaluation of *Ehrad52* epistasis group proteins regulation in response to DNA damage will help us to better understand DNA repair by HR in *E. histolytica*. It seems that the molecular events related to DNA lesions produced by genotoxic agents can be barely inferred from gene expression profiling. Indeed, studies in yeast and mammals have shown no-relationship between genes whose expression is increased after different DNA-damaging treatments (ionizing radiation, UV light, cisplatin, H₂O₂) and those genes that are involved in protecting against cytotoxicity to the same agents [28,29].

We focused on *Ehrad51* gene because RAD51 proteins have been demonstrated as key players in recombinational DNA repair in lower and higher eukaryotes [for review see [12]]. Interestingly, the *Ehrad51* transcript steady state levels were about 15-fold higher at 30 min post-UV-C treatment and decreased 3 and 12 h later, suggesting that EhRAD51 could be participating in HR in the early steps of DNA repair. Similar transcriptional activation after UV treatment has been reported as a common characteristic for recA/rad51 homologs of *Tetrahymena thermophila* [30] and *Halobacterium sp*. [31]. In agreement with the RT-PCR results, Western blot assays showed a dramatic increase of EhRAD51 in cytoplasm and nucleus, 30 min after DNA breaks were introduced into the *E. histolytica* genome. The fact that specific polyclonal antibodies immunodetected a 46 kDa EhRAD51 protein suggest that some posttranslational modifications of the cytoplasmic 41 kDa EhRAD51 could be a requirement for its translocation to the nucleus where DNA repair takes place. Taking in consideration that the *Ehrad51* sequence lacks a nuclear localization signal, an alternative possibility might be that EhRAD51 needs to interact with other protein(s) to be transported inside the nucleus. However, additional experiments are required to corroborate these hypotheses.

As observed for yeast and human homologs [32], laser confocal microscopy evidenced focal sites of the EhRAD51 protein scattered in the nucleus at 30 min and 3 h after DNA damage. Congruently, the EhRAD51 nuclear foci-like structure occurrence was consistent with the DNA fragmentation degree observed in TUNEL and neutral comet assays. Since UV-C treatment did not affect trophozoites viability, it is tempting to suggest that DNA repair mechanisms involving EhRAD51 foci formation were activated to restore genome integrity after genotoxic insult.

In silico analysis demonstrated that the predicted EhRAD51 protein contains all functional and structural motifs that are important for RECA/RAD51 recombinases activities. To experimentally support its role in DNA repair by HR, we performed the basic characterization of EhRAD51 protein. EhRAD51 functional properties were
similar to those previously reported for RAD51 homolo-
gous [33-35]. EhRAD51 was able to bind both ssDNA and
dsDNA substrates in the presence of ATP and Mg^2+. The
various rEhRAD51-DNA complexes may be related to dif-
ferent amounts of rEhRAD51 molecules bound to ssDNA
or dsDNA probe. Finally, EhRAD51 promoted specific
three-stranded pairing structure formation or D-loop.

Based on the data presented here, we proposed a working
model for DNA DSB repair involving the EhRAD51
recombinase. When a DSB is introduced in *E. histolytica*
geno, EhH2AX histones become phosphorylated,
which could induce chromatin remodeling and accumu-
lation of the EhRAD52 epistasis group proteins at the
DNA DSB site. We observed that EhRAD51 was relocated
into the DNA repair nuclear foci, where it could mediate
DNA paring and homologous strand exchange to restore
genome integrity. It is also possible that *E. histolytica*
RAD51 protein may play a role in genome rearrangements
that naturally occur within this organism during DNA
synthesis. Therefore, it will be interesting to evaluate its
involvement in frequent ploidy changes, unscheduled
gene amplification and duplication events observed in *E.
histolytica* genome [3,4]. Our next challenge will involve
studying *in vivo* HR and the relevant role of EhRAD51 in
this process in *E. histolytica*.

**Conclusion**

Our results provide the first data supporting the role of the
RAD52 epistasis group genes in DNA repair process in *E.
histolytica*. We showed that *E. histolytica* RAD52 epistasis
group genes, were differentially expressed when DNA
fragmentation was induced by UV-C irradiation. We also
showed that EhRAD51 protein was overexpressed and
relocalized in nuclear foci-like structures after DNA dam-
age, and demonstrated that recombinant EhRAD51 func-
tion as a recombinase *in vitro*. These data evidenced a
potential role of EhRAD51 protein in DNA damage
response in this ancient eukaryotic parasite.

**Methods**

**E. histolytica cultures**

Trophozoites of *E. histolytica* clone A (strain HM1: IMSS)
were axenically cultured in TYI-S-33 medium [36] at 37°C
and harvested during exponential growth phase.

**Trophozoites UV-C light irradiation**

Trophozoites (2 × 10^6) grown in culture bottles were
transferred into glass dishes and incubated at 37°C for 30
min. Medium and floating cells were discarded, and
adhered trophozoites were irradiated with 254 nm UV-C
light at 150 J/m² for 8 s using a UV Stratagene 1800
device (Stratagene). After treatment, cells were incubated
in fresh TYI-S-33 medium at 37°C for 0.5, 3, 6 and 12 h
to be used in different experiments. Non-irradiated cells
were used as a control in all experiments. Cell viability

**Evaluation of DNA fragmentation by TUNEL assay**

Trophozoites (2 × 10^6) were harvested at 0.5, 3, 6 and 12
h after UV-C irradiation, washed with PBS 1× and fixed
with 1% paraformaldehyde. After cell permeabilization
with 70% ethanol, DNA damage was quantified using the
APO-BrdUTP TUNEL Assay Kit (Molecular Probes) in
order to detect 3'-hydroxyl ends in DNA. Permeabilized
trophozoites were incubated at 37°C for 1 h in the DNA-
labeling solution, which contains terminal deoxynucleoti-
dyl transferase enzyme (TdT) and deoxynucleotidyl analog
5-bromo-2’-deoxyuridine 5’-triphosphate (BrdUTP).
Then, cells were washed twice and suspended in antibody
staining solution (Alexa Fluor 488 dye-labeled anti-BrdU
antibody) at room temperature for 1 h. After that, cells
were incubated in propidium iodide/Rnase A staining
buffer at room temperature for 30 min. Samples were ana-
lyzed by flow cytometry in a BD FACS Calibur system and
fluorescence data were plotted with the FloJo software.

**Evaluation of DNA fragmentation by Comet assay**

Trophozoites (5 × 10^6) were harvested at 0.5, 3, 6 and 12
h after UV-C irradiation. Neutral comet assay were per-
formed using protocols from Tice and co-workers [37].
Briefly, cells were mixed with agarose and spread over a
warmed, precoated microscope slides. Agarose was
allowed to solidify at 4°C, followed by immersion in cold
lysis fresh solution (2.5 M NaCl, 100 mM EDTA, 10 mM
Tris, pH 7) overnight. Next, electrophoresis was carried
out in neutral buffer for 20 min at 1.5 V/cm (measured
electrode to electrode) in the dark at 4°C. Finally, the
slides were completely dried and ethidium bromide-
stained DNA was observed at 400× magnification using an
epifluorescence microscope (Leica DMIL).

**Detection of phosphorylated EhH2AX histones**

The two *Ehh2ax* genes, which are homologous to the
human h2ax gene, had been previously reported [22]. Their
existence in the *E. histolytica* Pathema database [38] were
confirmed by BLAST using yeast H2A and human H2AX
protein sequences as queries. The presence of phosphor-
ylated forms of EhH2AX histone (γEhH2AX) in *E. histolyt-
ica* protein extracts obtained 10 or 30 min after UV-C
irradiation was evaluated by Western blot assays using the
anti-phospho-Histone H2AX (pSer139), which was devel-
oped in rabbit using a synthetic phosphorylated peptide
corresponding to 134–142 aa residues (including the
phosphorylated Ser) of human H2AX histone C-terminus
(Sigma). Subcellular fractionation to obtain CE and NE
from clone A trophozoites was performed as described [39].
Proteins were separated by 10% SDS-PAGE, trans-
ferred to nitrocellulose membranes (BioRad) and blocked
with 1% BSA/PBS solution. Then, filters were incubated at
room temperature for 2 h with the anti-human γH2AX polyclonal antibody (1:7000 dilution), washed with PBS 1× 0.05% Tween and incubated at 37 °C for 1 h with goat anti-rabbit IgG horseradish peroxidase secondary antibody (Zymed) at 1:10000 dilution. Bands were revealed by ECL Plus Western blotting system (Amersham). As internal controls, we used polyclonal antibodies (1:1000 dilution) raised against the E. histolytica poly(A) polymerase EhPAP and anti-actin antibodies.

**In silico identification of E. histolytica genes homologous to yeast RAD52 epistasis group**

RAD52 epistasis group related genes were identified in E. histolytica Pana database using both yeast and human protein sequences as queries. Putative E. histolytica orthologous proteins were selected from BLAST analysis according to the following criteria: (i) at least 20% identity and 35% homology to the query sequence; (ii) e-value lower than 0.002; and (iii) absence of stop codons in the coding sequence. Predicted aa sequences were aligned by the ClustalW software [40]. Functional domains were predicted by the Prosite program [41]. Phylogenetic inference was performed using the Neighbor-joining distance method [42] as implemented in the Molecular Evolutionary Genetics Analysis (MEGA version 3.1) software [43]. Tree robustness was established by bootstrapping test, involving 1000 replications of the data based on the criteria of 50% majority-rule consensus.

**RT-PCR assays**

Total RNA was obtained using Trizol reagent (Invitrogen) from trophozoites of clone A grown in basal culture conditions or after UV-C treatment. Semi-quantitative RT-PCR was performed as previously described [44] using 1 μg of total RNA and specific primers for each gene (Table 2). As a control, we amplified a 25S rRNA gene internal sequence. Products were separated by 6% PAGE, stained with ethidium bromide and submitted to densitometric analysis in a Gel doc 1000 apparatus (BioRad) using the Quantity One software. Data are the mean of three independent assays.

**Cloning of the Ehrad51 gene**

The 1098-bp full-length Ehrad51 gene was PCR-amplified from genomic DNA of clone A trophozoites using Ehrad51-S (5'-CGGGATCCAAAGTAGTAGAGTGGCAAGCA-3') sense and Ehrad51-AS (5'-CCAAGCTTGCAATCTCCCTCCATATTG-3') antisense primers, which contain Bam HI and Hind III restriction sites, respectively (underlined). Amplification was performed as follows: 94 °C for 5 min and 30 cycles at 94 °C for 35 s and 72 °C for 1 min, plus a final extension step at 72 °C for 7 min, using High Fidelity DNA Taq polymerase (Invitrogen). The PCR product was purified and cloned in frame into the pRSET A expression vector (Invitrogen). The recombinant pRSET-Ehrad51 plasmid construct was confirmed by automated DNA sequencing in an ABI-PRISM 310 (Applied Biosystem) sequencer.

**Expression and purification of recombinant EhRADS1 (rEhRADS1) protein**

E. coli BL21 (DE3) pLysS bacteria were transformed with pRSET-Ehrad51 plasmid and grown at 37 °C in 2-TY medium containing 100 μg/ml ampicillin and 34 μg/ml chloramphenicol to an OD600nm of 0.6. The expression of rEhRAD51 was induced with 1 mM isopropyl beta-D-thiogalacto pyranoside (IPTG) at 37 °C for 3 h. Cells were harvested, resuspended in lysis buffer (50 mM NaH2PO4, 300 mM NaCl, 10 mM imidazole, pH 8.0) and lysed by sonication at 4 °C. Soluble rEhRAD51 was purified near to homogeneity under denaturing and native conditions through Ni2+-NTA affinity chromatography according to the manufacturer recommendations (Qiagen). Purified rEhRAD51 identity and integrity were confirmed by 10% SDS-PAGE and Western blot assays using anti-6xHis tag antibodies (Roche) at 1:5000 dilution and the ECL Plus Western blotting detection system (Amersham).

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**Table 2: Primers used in RT-PCR assays**

| Gene          | Sense primer       | Antisense primer       | Amplified product (bp) | Tm (°C) |
|---------------|--------------------|------------------------|------------------------|---------|
| Ehmre1I       | 5’-CGAGAAGAAGACACGCTCAAA  | 5’-CTTTCCTTTTTCTTCACGCAA | 391                     | 49.5    |
| Ehrad50       | 5’-CAGCAGAAGAAGATCTCAAA  | 5’-CTGCATAATTGTTGTGGCAA | 277                     | 49.5    |
| Ehhs1         | 5’-CACCCTCTCCACCCACACAGTAT  | 5’-CTCCACCAATGATGACCAT | 549                     | 49.0    |
| Ehrad51       | 5’-ATTGCTTTTACACAAAG  | 5’-TTTCTTCTGAAATTAAAC | 400                     | 49.5    |
| Ehrad51/C     | 5’-CCACATGCTTTTGGGTGCT  | 5’-GAATTATCCGATGAAATGCT | 155                     | 45.0    |
| Ehrad52       | 5’-ATGACTGAAATAGATACCTC  | 5’-AATTTGATTGTTTTAAGAAT | 730                     | 37.5    |
| Ehrad54       | 5’-GTCATGCATACATCCCAAGAATTAATTA  | 5’-TCACAATCCTTCCTCGATTG | 499                     | 47.5    |
| Ehrad54b      | 5’-GGCCAGGAAAATATTCCTAATAGC  | 5’-GTGGATCTGCTGGATTCATAAGTC | 876                     | 50.0    |
| Ehrad52/22    | 5’-ATGCTCTGATGAAAATACCAATACCCAC  | 5’-TCATTTCCTGATGCTTACATTACT | 516                     | 44.5    |
| 25S rRNA      | 5’-TATCAATCAAGACACCCCGCT  | 5’-AAAAGA AAAACTAAGCCCAGTAA | 631                     | 51.0    |
| actin         | 5’-AGCTGTTCTTTTCTATATGC  | 5’-TTCTTCTGACGACTATGATGTT | 220                     | 48.0    |
Production of polyclonal antibodies raised against EhRAD51

Purified rEhRAD51 was submitted to preparative 10% SDS-PAGE, electroeluted from Coomassie stained-gels and subsequently used as antigen to immunize a New Zealand male rabbit. An initial dose of 200 μg of rEhRAD51 in complete Freund’s adjuvant (Sigma) was subcutaneously inoculated into the animal, and then three doses of 100 μg in incomplete Freund’s adjuvant were injected every 15 days. One week after the last immunization, the rabbit was bled and polyclonal antiserum was obtained. IgGs were purified through protein G sepharose chromatography and tested for reactivity against rEhRAD51 protein by Western blot assays.

Immunodetection of EhRAD51 in subcellular extracts

Western blot assays were performed using CE and NE proteins obtained before or 30 min after UV-C irradiation, and the membranes were incubated with anti-EhRAD51 polyclonal antibodies (1:1000 dilution) and goat anti-rabbit IgG horseradish peroxidase secondary antibody (Zymed) (1:10000 dilution). Immunodetected proteins were revealed with the ECL Plus Western blotting system (Amersham). The specificity of the anti-EhRAD51 antibodies was confirmed using anti-EhRAD51 antibodies used as heterologous dsDNA control. Reactions were stopped by addition of 0.1% SDS. To prevent that EhRAD51 binds and shifts the ppg200 probe, samples were deproteinized with proteinase K (1 mg/ml) at 37°C for 10 min. Then, homologous dsDNA plasmid (1 μM) was added and the mixture was incubated at 37°C for 30 min. A non-related plasmid was used as heterologous dsDNA control. Reactions were performed substituting purified rEhRAD51 by the mock purified fraction obtained from untransformed bacteria. DNA-protein complexes were resolved on 6% non-denaturing TBE polyacrylamide gels, vacuum-dried and exposed to Phosphor Imager screen (BioRad).

D-loop structure formation assay

The EhRAD51 homologous DNA strand transfer activity was evaluated by the D-loop formation assay according to the described procedure [47]. A ssDNA fragment of 200 bases (ppg200), which is complementary to the 3'-UTR EhPgp5 gene cloned in the dsDNA plasmid [44], was [γ-32P]dATP (500 μCi/mmol) 3'-end labeled by T4 polynucleotide kinase at 37°C for 30 min. Increasing amounts of rEhRAD51 (0, 2.5, 5 and 7.5 μg) were pre-incubated in reaction buffer (50 mM Tris-HCl pH 7.8, 1 mM DTT, 10 mM MgCl2 and 1 mM ATP) with the ppg200 probe (10,000 cpm) at 37°C for 15 min. Then, homologous dsDNA plasmid (1 μM) was added and the mixture was incubated at 37°C for 30 min. A non-related plasmid was used as heterologous dsDNA control. Reactions were performed substituting purified rEhRAD51 by the mock purified fraction obtained from untransformed bacteria. DNA-protein complexes were resolved on 6% non-denaturing TBE polyacrylamide gels, vacuum-dried and exposed to Phosphor Imager screen (BioRad).

Authors’ contributions

MLC carried out most of the experiments and drafted the manuscript. EO participated in the study design, data interpretation and co-wrote the manuscript. Most of experiments presented here were performed in EO laboratory (CINVESTAV-IPN). LAM participated in the study design, data interpretation and bioinformatic analysis. CLC conceived the project, cloned the Ehrad51 gene, supervised the experiments and co-wrote the manuscript. All authors read and approved the final manuscript.
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