The knockdown of each component of the cysteine proteinase-adhesin complex of *Entamoeba histolytica* (EhCPADH) affects the expression of the other complex element as well as the *in vitro* and *in vivo* virulence

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SUMMARY

Entamoeba histolytica is the protozoan parasite causative of human amoebiasis, disease responsible for 40 000–100 000 deaths annually. The cysteine proteinase-adhesin complex of this parasite (EhCPADH) is a heterodimeric protein formed by a cysteine protease (EhCP112) and an adhesin (EhADH) that plays an important role in the cytopathic mechanism of this parasite. The coding genes for EhCP112 and EhADH are adjacent in the *E. histolytica* genome, suggesting that their expression may be co-regulated, but this hypothesis has not yet been confirmed. Here, we performed the knockdown of EhCP112 and EhADH using gene-specific short-hairpin RNAs (shRNA), and the effect of these knockdowns on the expression of both complex components as well as on the *in vitro* and *in vivo* virulence was analysed. Results showed that the knockdown of one of the EhCPADH components produced a simultaneous downregulation of the other protein. Accordingly, a concomitant reduction in the overall expression of the complex was observed. The downregulation of each component also produced a significant decrease in the *in vitro* and *in vivo* virulence of trophozoites. These results demonstrated that the expression of EhCP112 and EhADH is co-regulated and confirmed that the EhCPADH complex plays an important role in *E. histolytica* virulence.

Key words: *Entamoeba histolytica*, EhCPADH complex, virulence, downregulation, RNA interference, shRNA, expression co-regulation.

INTRODUCTION

Amoebiasis, is caused by the protozoan parasite *Entamoeba histolytica*. This parasite infects approximately 50 million individuals and provokes 40 000–100 000 deaths annually (Ximenez *et al*. 2009). During infection, *E. histolytica* colonizes the mucosal surface of the large intestine, which can be asymptomatic or result in diarrhoea or colitis. In addition, trophozoites have the capacity to spread to other organs, such as liver, lung, genitals, spleen, skin and brain (Ali *et al*. 2008). The cytopathic mechanism of this parasite consists of three main steps: adherence, cytolysis and phagocytosis (Ravdin *et al*. 1980) and several molecules that participate in these events have been reported (Faust and Guillen, 2012).

The *E. histolytica* cysteine proteinase-adhesin (EhCPADH) complex, a surface membrane and vacuolar heterodimeric *E. histolytica* protein complex formed by a cysteine protease (EhCP112, 446 amino acids) and an adhesin (EhADH, 687 amino acids), has been shown to be involved in the cytopathic mechanism of this parasite (Arroyo and Orozco, 1987; Garcia-Rivera *et al*. 1999; Betanzos *et al*. 2013). *In vitro*, a recombinant EhCP112 polypeptide digests gelatin, type I collagen, fibronectin and hemoglobin, as well as binds to red blood cells and destroys Madin Darby Canine Kidney (MDCK) cell monolayers (Ocadiz *et al*. 2005). On the other hand, EhADH has a cell-adherence domain at its carboxy-terminus (Martinez-Lopez *et al*. 2004), and at the amino-terminus it contains a Bro-1 domain, a sequence that binds with multivesicular body components such as yeast Snf7 and mammalian CHMP4b, and can function to target EhADH to endosomes (Banuelos *et al*. 2005). Interestingly, the subcutaneous immunization of

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hamsters with a recombinant polypeptide containing the last 243 amino acids of the EhADH carboxy-terminus (rEhADH243) as well as DNA vaccination with a mixture of *Ehcp112* and *Ehadh* genes decreased the formation of hepatic abscesses (Madriz *et al*. 2004; Martinez-Lopez *et al*. 2004; Martinez *et al*. 2009). Moreover, genes encoding EhCP112 and EhADH are located adjacent to each other in the *E. histolytica* genome, separated by only 188 bp (Garcia-Rivera *et al*. 1999). This close gene proximity suggests a possible co-regulation in their expression, although this assumption has not been experimentally tested.

For this parasite, gene knockout technology is not currently feasible. However, multiple gene knockdown approaches have been developed, including antisense expression (Sahoo *et al*. 2003) and a number of RNA interference (RNAi)-based methods (Solis and Guillen, 2008; Linford *et al*. 2009). One variant of the RNAi-based gene knockdown methods is the use of double-stranded short-hairpin RNAs (shRNA), which has been shown to produce efficient, specific and long-lasting gene silencing (Deng *et al*. 2014). In a previous study, we have shown that the transitory downregulation of *Ehcp112* in *E. histolytica* trophozoites by the small interfering RNA (siRNA) method produces a significant reduction of the *in vitro* virulence (Ocadiz-Ruiz *et al*. 2013). *Ehcp112* knockdown by siRNA only lasted 16 h, making not possible to measure the *in vivo* virulence of the silenced trophozoites. On the other hand, the effect of *Ehcp112* downregulation on the *Ehadh* gene expression, as well as the reciprocal effect of *Ehadh* knockdown on *Ehcp112* expression, and on trophozoites virulence have not yet been investigated.

In the present study, we investigated the effect of EhCP112 and EhADH knockdown by shRNA interference on the expression of the EhCPADH gene and protein complex and on *E. histolytica* virulence. Results indicated that the downregulation of each component of the complex resulted in reduced expression of the other constituent of the complex, and therefore, an overall decrease in EhCPADH levels. Knockdown also produced a decrease in the *in vitro* and *in vivo* virulence of trophozoites. Taken together, results support the hypothesis that trophozoites possess a mechanism for the co-regulation of EhCP112 and EhADH expression; and that the EhCPADH complex plays an important role in *E. histolytica* virulence.

MATERIAL AND METHODS

Cell cultures

Trophozoites of the virulent HM1:IMSS strain were axenically cultured in TYI-S-33 medium and harvested during the logarithmic growth phase as previously described (Diamond *et al*. 1978).

Madin Darby Canine Kidney (MDCK) epithelial cells were grown until confluence in 24-well plates (Corning, Sigma-Aldrich, St. Louis Mo, USA) in Dulbecco's Modified Eagle's Medium (DMEM) (Gibco Life Technologies, Grand Island, NY, USA) supplemented with penicillin (100 i.u. mL^{-1}; Invitrogen, Mexico), streptomycin (100 mg mL^{-1}; Invitrogen, Mexico), and 10% fetal bovine serum (Gibco Life Technologies, Grand Island, NY, USA).

shRNA design and construction

The *Ehcp112* and *Ehadh* genes were analysed by the online Target Finder program (Ambion, Austin Tx, USA) to design the interfering RNAs. This analysis was performed to detect sequences of 29 nucleotides and with a guanine-cytosine content between 30 and 50%. In order to identify specific target sequences, putative interfering motifs were analysed using the Basic Local Alignment Search Tool (BLAST) program on the *E. histolytica* genome database (http://amoebadb.org). A unique sequence for each target transcript was then selected (Supplementary material, Table S1). Finally, DNA sequences containing the 29-nucleotide target motif, a 9-nucleotide loop and the complementary nucleotides to the target sequence (to produce a hairpin RNA) were cloned downstream of the U6 promoter in a vector containing ampicillin and hygromycin resistance genes (Linford *et al*. 2009). As a negative control we cloned a sequence coding for a shRNA with a unique non-related sequence (NRS).

shRNA transfection

To transfect trophozoites, 100 μg of each construct were first incubated at room temperature (RT) for 2 h with 10 μL of lipofectamine reagent (Turbofect Fermentas, Hanover, MD, USA), followed by exposure of 1×10^5 trophozoites to the shRNA/reagent mixture in serum-free amoeba medium for 4 h at 37 °C. Following incubation, the transfection medium was removed, and fresh supplemented medium was added and trophozoites were incubated at 37 °C for 48 h. After this time, selection of transfectants was then carried out by incubation of cells in medium containing 10 μg mL^{-1} of hygromycin (Invitrogen, Mexico).

Production of antibodies against EhADH

To generate antibodies against EhADH, its full-length gene was cloned into the EcoR1 and BamH1 restriction sites of the pVac1-msc vector (InvivoGen, San Diego, CA, USA). Then, female Balb/c mice of 4 weeks old were intramuscularly immunized each 7 days during 4 weeks with 10 ng of the pCvac1-EhADH construct.

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Western blot assays

Total extracts of *E. histolytica* trophozoites, obtained in the presence of protease inhibitors (Complete Mini, Roche-Mannheim, Germany), were separated by sodium dodecyl sulfate-10% polyacrylamide gel electrophoresis (SDS-10% PAGE) and transferred to nitrocellulose membranes. Membranes were then incubated with polyclonal antibodies against recombinant EhCP112 (Ocadiz-Ruiz *et al.* 2013) (1:2000) or EhADH (1:5000), or a monoclonal antibody against the EhCPADH complex (1:1000), followed by incubation with the respective secondary antibodies conjugated to horseradish peroxidase (Invitrogen, Mexico) (1:10 000). Antibody detection was achieved by incubation with the chromogen 3,3′-Diaminobenzidine tetrahydrochloride (Sigma-Aldrich, St. Louis, Mo, USA) and H_2O_2. As an internal loading control, membranes were exposed to anti-actin antibodies (1:20 000) (kindly provided by Dr Jose Manuel Hernandez-Hernandez at CINVESTAV-IPN, Mexico). Bands detected by the antibodies were analysed by scanning densitometry and data were normalized to actin content according to anti-actin antibody reactivity. For quantitative protein comparisons, trophozoites transfected with the non-related control sequence was taken as 100%.

Quantitative reverse-transcriptase polymerase chain reaction (qRT-PCR)

Total RNA was extracted from *E. histolytica* trophozoites using the TRIzol reagent (Invitrogen, Mexico). RNA was treated with DNase (Qiagen, Mexico) and 1 μg of RNA was reverse-transcribed using SuperScriptII and oligo dT (Invitrogen, Mexico). The resulting cDNA was used to perform quantitative PCR assays for *Ehcp112* and *Ehadh* transcripts using specific oligolnucleotides (online Supplementary material, Table S2), where the *Ehgapdh* gene was used as a normalization control. For each reaction the SYBR Green PCR Mastermix was used following the manufacture's protocol (Applied Biosystems) in a StepOnePlus Real-Time thermal cycler (Applied Biosystems). Cycling conditions for qRT-PCR assays were: 95 °C for 15 min, followed by 40 cycles of 95 °C, 30 s; 59 °C, 30 s; 72 °C, 45 s and one cycle of 72 °C for 3 min. At the end of each series, a melt curve was performed from 70 to 95 °C, increasing 0·2 °C at each cycle with a 5 s hold. The CT values were averaged for each oligonucleotide pair for each set of technical replicates, and sample values were normalized to the housekeeping gene *Ehgapdh*. The differences in gene expression were calculated using the comparative $\Delta\Delta$ C(t) method (Livak and Schmittgen, 2001). cDNA from three independent samples were examined in triplicate and statistical analysis was performed by analysis of variance (ANOVA) using GraphPad 6.

Immunofluorescence

Entamoeba histolytica trophozoites grown on sterile coverslips were fixed with 4% paraformaldehyde at 37 °C for 1 h and permeabilized with 0·5% (v/v) Triton X-100 for 1 min at RT. Non-specific binding sites were blocked with 1% (w/v) bovine serum albumin (BSA) for 20 min at RT. Subsequently, trophozoites were incubated with primary antibodies (rabbit anti-EhCP112 or mouse anti-EhADH) overnight at 4 °C and then, with their respective secondary antibodies (rhodamine-coupled anti-rabbit immunoglobulin G (IgG), or fluorescein-coupled anti-mouse IgG) (Sigma-Aldrich, St. Louis Mo, USA) (1:1000) for 1 h at 37 °C. In addition, nuclei were counterstained with 30 nM of 4′,6-Diamidino-2-Phenylindole (DAPI) (Invitrogen, Mexico) for 15 min at RT. Finally, samples were placed in fluorescence mounting medium (Vectashield, Vector Laboratories, Burlingame, CA, USA) and examined with a confocal microscope (Carl Zeiss LSM 700). Observations were performed in 14 planes from the top to the bottom of each sample, and the distance between scanning planes was 1 μM. To quantitatively assess the fluorescence intensity, the images were analysed in the CellProfiler program (http://www.cellprofiler.org) using three images per group, with the standard intensity value for each channel being recorded and analysed in GraphPad prism 6 using two-way ANOVA to compare intensity between EhCP112- or EhADH-downregulated trophozoites with control trophozoites (transfected with NSR) as the statistic tool with significance value of $P \leqslant 0{\cdot}05$.

Cytopathic and cytotoxic assays

Cytopathic assays, defined as the ability of live trophozoites to destroy cultured cells (Lushbaugh *et al.* 1978), and cytotoxic assays, described as the ability of amoebic extracts to destroy cultured cells (Orozco *et al.* 1978) were carried out as previously described (Rodriguez and Orozco, 1986). Briefly, MDCK cell monolayers (1×10^5 cells) were incubated for 2 h at 37 °C with 1×10^5 trophozoites for cytopathic assays or with total extracts from 1×10^6 trophozoites plus 0·02% of 2-mercaptoethanol for cytotoxic assays. Then, trophozoites or their extracts were eliminated by washing with phosphate-buffered saline (PBS). Remaining cells were fixed with 2·5% (v/v) glutaraldehyde and stained with 1% (w/v) methylene blue. After exhaustive washes, the dye captured by cells was extracted with 0·1 M hydrogen chloride and measured in a spectrophotometer (Beckman Coulter DU800) at 660 nm. Results

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were reported as the mean ± S.D. of three independent experiments, each run in triplicate. Statistical analysis was performed by ANOVA using GraphPad 6.

Erythrophagocytosis

Erythrophagocytosis was performed using human red blood cells (hRBCs) as previously described (Voigt *et al*. 1999). Briefly, trophozoites suspended in serum-free culture medium were incubated with fresh hRBCs (O Rh+) from healthy donors (1:100 ratio) at 37 °C with slight agitation for 10 min (Orozco *et al*. 1983). Non-ingested erythrocytes were lysed by incubation in distilled water for 10 min at RT. This was followed by extensive washing and treatment of trophozoites with 1 mL of concentrated formic acid (J.T. Baker, Mexico), during which the parasites, with internalized hRBCs, are lysed. Phagocytosis rates were determined by measuring the haemoglobin released by the ingested erythrocytes (absorbance at 405 nm against a formic acid blank using a Beckman Coulter DU800 spectrophotometer). Data were normalized using the formula A_{405} of sample/A_{405} of control (trophozoites transfected with the NRS). Results were reported as the mean ± S.D. of normalized values from three independent experiments performed in triplicate. Statistical analysis was performed by ANOVA using GraphPad 6.

Adherence

Trophozoite adherence was evaluated using hRBCs as previously described (Garcia-Rivera *et al*. 1982). Briefly, parasites suspended in serum-free culture medium were incubated with fresh hRBCs (O Rh+) from healthy donors (1:100 ratio) at 4 °C with slight agitation for 10 min. Samples were then fixed with 2·5% (v/v) glutaraldehyde and erythrocytes were stained with 3% 3,3′-Diaminobenzidine tetrahydrochloride and H_2O_2. Finally, after exhaustive washes with PBS, the number of hRBCs attached to 100 trophozoites was counted. These experiments were performed in three independent experiments in triplicate. For graphic purposes, the number of adherent erythrocytes in control parasites was considered as 100% efficiency. Statistical analysis was performed by ANOVA using GraphPad 6.

In vivo *virulence assays*

For *in vivo* virulence experiments, groups of six male Syrian golden hamsters (*Mesocricetus auratus*) were directly challenged in the right lobule of the liver by injection with 1×10^6 virulent untransfected trophozoites (strain HM1:IMSS) or those transfected with the shRNAs for EhCP112, EhADH or the NRS (specificity control). Seven days after challenge, animals were sacrificed under anaesthesia and liver damage was calculated as the weight of abscesses formed divided by the weight of the complete liver before injured areas were removed. Statistical analysis was performed by ANOVA using GraphPad 6. All animals used in this study were handled in accordance with the guidelines of the Institutional Animal Care and Use Committee. Our institution fulfils all the technical specifications for the production, care and use of laboratory animals and it is certified by national law (NOM-062-ZOO-1999).

RESULTS

Knockdown of one element of the EhCPADH complex decreased the expression of the other component

To analyse the knockingdown effect of each protein comprising the EhCPADH complex on the expression of the other protein member and expression of the overall complex, we carried out the downregulation of EhCP112 and EhADH using a shRNA interference approach. A specific 29-nt sequence, unique in the *E. histolytica* genome for each gene was chosen for the knockingdown of each component of the EhCPADH complex. These sequences (online Supplementary material, Table S1) were used to produce DNA fragments that were cloned in an episomal vector-based system, which uses the *E. histolytica* U6 promoter, to drive the expression of the shRNAs (Linford *et al*. 2009). Then, to check the downregulation efficiency, we performed Western blot assays on trophozoites grown for 3 months in the presence of hygromycin. Antibodies against EhCP112 recognized two bands of 49 and 36 kDa (Fig. 1A and B) in trophozoites transfected with the NRS control. These molecular weights correspond to the pro-enzyme and the mature enzyme, respectively (Ocadiz-Ruiz *et al*. 2013). Antibodies against EhADH detected a single 75 kDa band (Fig. 1A and B), corresponding to the expected molecular weight for this polypeptide. However, in shRNA-treated trophozoites we observed a decreased expression of EhCP112 and EhADH regardless of the specific shRNA sequence used (Fig. 1A and B; EhCP112-, EhADH-). Densitometric analyses confirmed the decrease in expression of both polypeptides (Fig. 1C and D). In trophozoites transfected with the shRNA for EhCP112 (EhCP112(-)), expression of this protease decreased to 5·8% with respect to the control (Fig. 1C). Interestingly, in these trophozoites EhADH protein levels also were reduced in expression to 58% of the NRS control (Fig. 1C). This phenomenon of downregulation of both EhCPADH components was also observed in trophozoites transfected with the shRNA for EhADH (EhADH(-)), which showed a decrease in the expression for EhADH (38·5% of NRS) and EhCP112 (26·9% of NRS) (Fig. 1D). These results indicate a co-regulated

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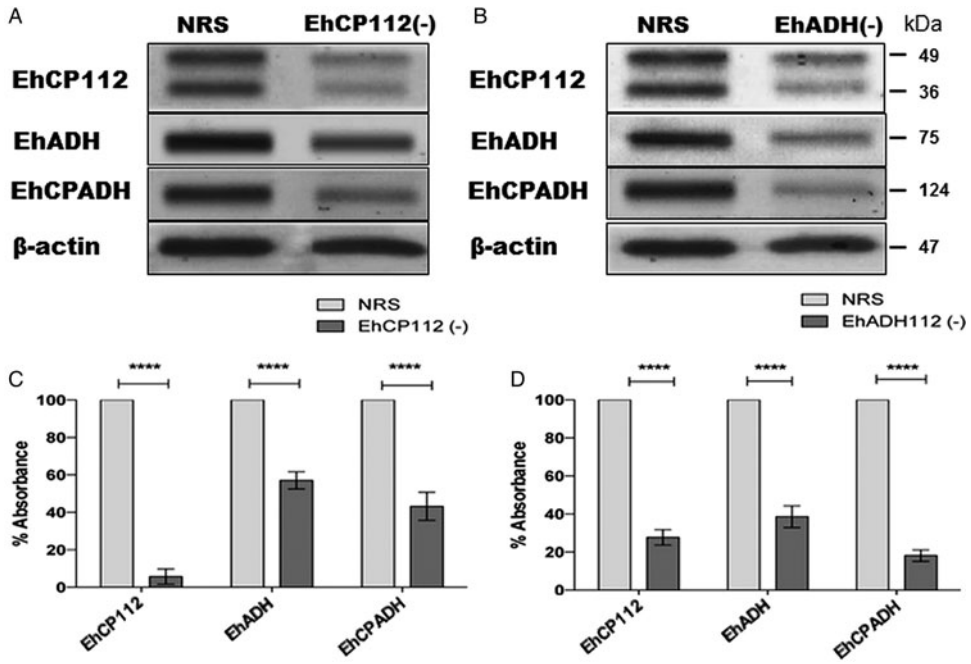

Fig. 1. Knockdown of EhCP112 and EhADH. (A, B) Western blot. Total proteins of *E. histolytica* trophozoites transfected with plasmids expressing shRNAs for knockdown EhCP112 (EhCP112(-)) (A) and EhADH (EhADH(-)) (B) were subjected to Western blot assays using antibodies against EhCP112, EhADH, and the complex itself. As an internal control, samples were probed with anti-actin antibodies. (C, D) Relative expression of EhCP112 and EhADH. Bands detected by antibodies in panels A and B were analysed by densitometry and values were normalized with those obtained from the internal control. Relative expression of each band in control trophozoites transfected with a NRS was taken as 100%. Values represented the mean ± S.D. of three independent experiments. (****) $P < 0{\cdot}001$. Abbreviations: EhADH, *Entamoeba histolytica* adhesin; EhCP112, *Entamoeba histolytica* cysteine protease complexed with EhADH; EhCPADH, Entamoeba histolytica complex formed by EhADH and EhCP112; NRS, non-related sequence; shRNAs, short-hairpin RNA.

expression of EhADH and EhCP112, because the knockdown of one of them produces also a downregulation of the other protein. This result suggests that the knockdown of these proteins may also affect the assembly of the EhCPADH complex. Indeed, the level of this complex, identified using a monoclonal antibody that recognizes the 124 kDa band corresponding to EhCPADH (Arroyo and Orozco, 1987), was also diminished in EhCP112(-) and EhADH(-) trophozoites (Fig. 1A and B). Expression level of the complex decreased to 42·3 and 17·3% of control NRS levels in EhCP112(-) and EhADH(-) trophozoites, respectively (Fig. 1C and D).

Knockdown of one of the EhCPADH element also decreases the mRNA level of the other component

To analyse whether the expression co-regulation of the proteins that form the EhCPADH complex is achieved also at transcriptional level we performed qRT-PCR assays on downregulated trophozoites using specific primers for each gene (online Supplementary material, Table S2). Results showed that in EhCP112(-) trophozoites the mRNA levels for *Ehcp112* decreased to 57% of control, whereas the *Ehadh* transcript was reduced to 60·9% of control (Fig. 2A). On the other hand, in EhADH(-) trophozoites, the mRNA level of *Ehadh* diminished to 64% and the *Ehcp112* transcript decreased to 75% with respect to control (Fig. 2B). The drop in transcription of both genes when one of them is knockingdown suggested that the mRNA amount of one component of the EhCPADH complex impacts the transcription of the other constituent, supporting the hypothesis that transcription of both genes is co-regulated.

Immunofluorescence of knockingdown trophozoites

Once that downregulation of EhCP112 and EhADH was confirmed, we performed immunofluorescence assays in order to determine EhCP112 and EhADH expression and localization in knockingdown trophozoites. In control cells, EhCP112 was observed as large discontinuous segments of the plasma membrane and in granules within the cytoplasm, whereas EhADH was detected mainly in a continuous pattern in the plasma membrane of trophozoites (Fig. 3A). Co-localization of both proteins in large segments of the plasma membrane indicates the presence of the EhCPADH complex at these sites (merged images, Fig. 3A). In contrast to controls, EhCP112(-) trophozoites displayed a significant reduction in both cytoplasmic and membrane anti-

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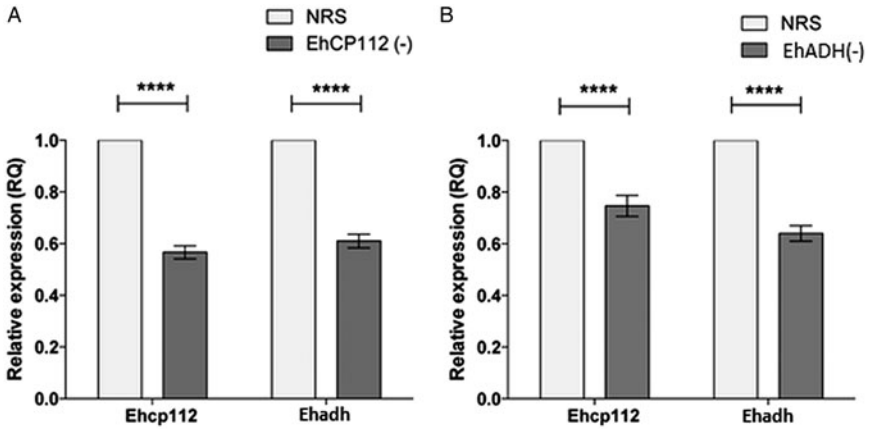

Fig. 2. mRNA levels of Ehcp112 and Ehadh in downregulated trophozoites. Total RNA from EhCP112(-) (A) and EhADH(-) (B) was subjected to qRT-PCR using specific primers for *Ehcp112* and *Ehadh* transcripts. Target transcript levels were normalized to the *gapdh* gene. Relative expression in trophozoites transfected with aNRS was taken as the unit (1·0). Values represented the mean ± S.D. of three independent experiments each performed in triplicate. (****) $P < 0{\cdot}001$. Abbreviations: EhADH, *Entamoeba histolytica* adhesin; EhCP112, *Entamoeba histolytica* cysteine protease complexed with EhADH; EhCPADH, Entamoeba histolytica complex formed by EhADH and EhCP112; NRS, non-related sequence; qRT-PCR, quantitative reverse-transcriptase polymerase chain reaction.

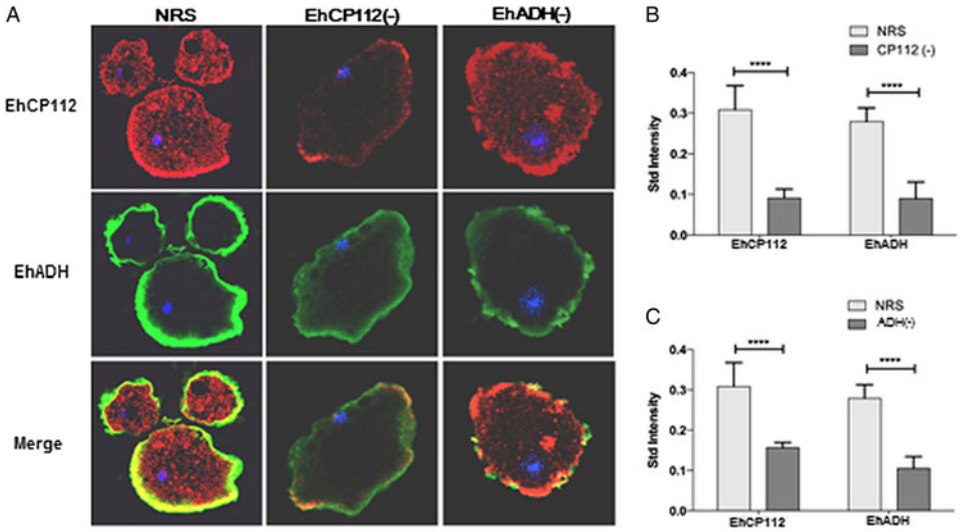

Fig. 3. Cellular localization of EhCP112 and EhADH in downregulated trophozoites. (A) Immunofluorescence. *Entamoeba histolytica* trophozoites transfected with plasmids expressing shRNAs for EhCP112 (EhCP112(-)), EhADH (EhADH(-)) and a NRS were fixed, permeabilized and incubated with rabbit anti-EhCP112 or mouse anti-EhADH antibodies, and then, with anti-rabbit IgG and anti-mouse IgG secondary antibodies coupled to rhodamine and fluorescein, respectively. Nuclei were counterstained with DAPI. After that, cells were observed through confocal microscopy; 100X magnifications. (B) Fluorescence intensity analysis. Intensity of fluorescence signals were analysed in the CellProfiler program, with the standard intensity value for each channel measured and analyzed. (****) $P < 0{\cdot}001$. Abbreviations: EhADH, *Entamoeba histolytica* adhesin; EhCP112, *Entamoeba histolytica* cysteine protease complexed with EhADH; EhCPADH, Entamoeba histolytica complex formed by EhADH and EhCP112; DAPI, Diamidino-2-Phenylindole; NRS, non-related sequence; shRNAs, short-hairpin RNA.

EhCP112 reactivity (Fig. 3A). Analysis of fluorescence intensity revealed a reduction to approximately 33% of control in the protein expression (Fig. 3B). In EhCP112(-) trophozoites, EhADH also was diminished to ~33% of control, with this protein being localized mainly in the plasma membrane (Fig. 3A and B). The merge images showed a co-localization of EhCP112 and EhADH in isolated areas of the plasma membrane (Fig. 3A). Similarly, EhADH(-) trophozoites showed a decrease in the fluorescence detection of EhADH of about 50% (Fig. 3A and C), with this protein taking on a patchy distribution within the plasma membrane (Fig. 3A). In EhADH (-) trophozoites, EhCP112 showed a notable decreasing in the detection of the protein (~33% of control level) (Fig. 3A and C).

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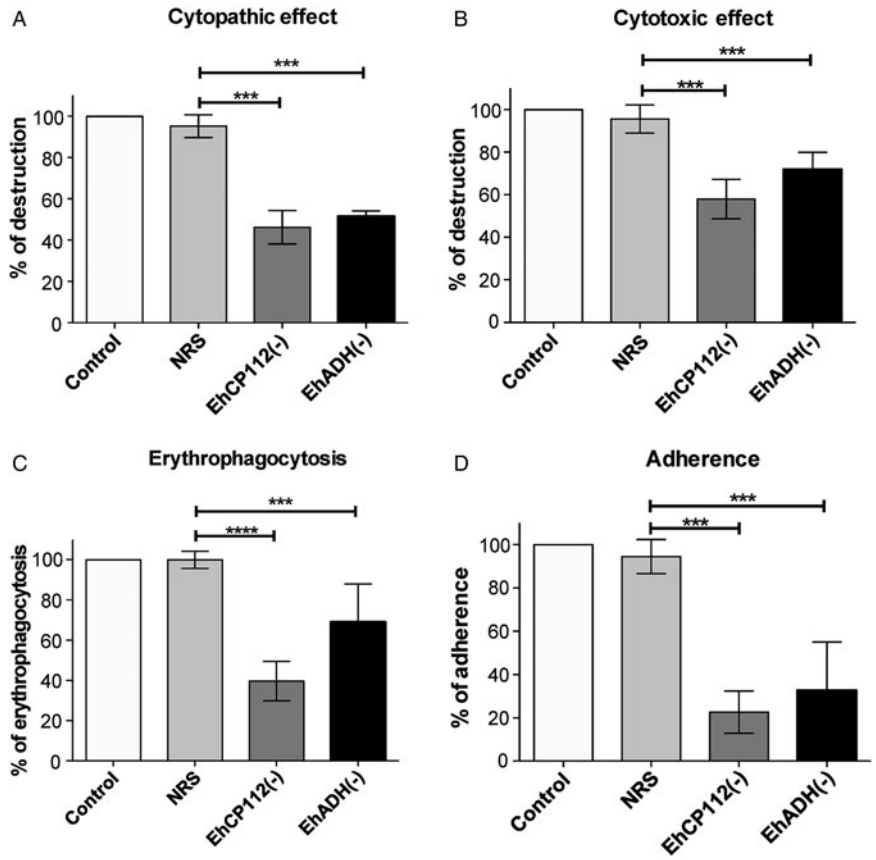

Fig. 4. *In vitro* virulence of EhCP112(-) and EhADH(-) trophozoites. Trophozoites non-transfected (control) or transfected with shRNA for NRS, EhCP112 or EhADH were tested for various *in vitro* virulence-related functions. (A) Cytopathic effect. Live trophozoites were incubated with MDCK monolayers for 2 h at 37 °C. Then, monolayers destruction was evaluated as described in the Materials and Methods section. Destruction by control trophozoites was taken as 100%. (B) Cytotoxic effect. Trophozoite extracts were incubated with MDCK monolayers for 2 h at 37 °C. Monolayers cytolysis was determined as described in the Materials and Methods section. Destruction by control trophozoites was taken as 100%. (C) Erythrophagocytosis. Trophozoites were incubated for 10 min with hRBC at 37 °C. Following lysis of non-ingested erythrocytes and exhaustive washes, trophozoites were lysed with formic acid and the released hemoglobin was measured by the absorbance at 405 nm. Data obtained with the control trophozoites were taken as 100% efficiency. (D) Adherence. Trophozoites were incubated for 10 min with hRBC at 4 °C. Following sample fixation, erythrocytes were stained with 3,3′-Diaminobenzidine + H_2O_2 and the number of erythrocytes attached to 100 trophozoites were counted. Adherence to control trophozoites was taken as 100% efficiency. All assays were performed in at least three independent experiments performed in triplicate. Results are expressed as the mean ± S.D. (***) $P < 0{\cdot}005$. (****) $P < 0{\cdot}001$. Abbreviations: EhADH, *Entamoeba histolytica* adhesin; EhCP112, *Entamoeba histolytica* cysteine protease complexed with EhADH; EhCPADH, Entamoeba histolytica complex formed by EhADH and EhCP112; hRBC, human red blood cells; MDCK, Madin Darby Canine Kidney cell line; shRNAs, short-hairpin RNA.

Knockdown of EhCP112 and *EhADH decreased the* in vitro *virulence*

To analyse the effect of the knockdown of EhCP112 and EhADH on trophozoite virulence we carried out cytopathic and cytotoxic assays on MDCK cell monolayers. In cytopathic assays, defined as the ability of live trophozoites to damage target cells, downregulated trophozoites showed a reduction in their ability to destroy cell monolayers in relation to the control. EhCP112(-) and EhADH(-) trophozoites destroyed 45·2 and 50·8%, respectively, of MDCK monolayers when compared with control trophozoites (Fig. 4A). Similarly, the cellular extracts of both knockingdown strains also exhibited a diminishing in their capacity to destroy MDCK cell monolayers (cytotoxic assays). In these experiments, exposure of monolayers to extracts of EhCP112(-) and EhADH(-) trophozoites resulted in reduced cytotoxic efficiencies of 57·7 and 71·5%, respectively, of control levels (Fig. 4B).

Another aspect analysed in downregulated trophozoites was their rate of phagocytosis, because it has been demonstrated that this activity is directly associated with the virulence mechanism of *E. histolytica* (Orozco *et al*. 1983). Phagocytosis experiments were carried out by incubating hRBC with knockingdown and control trophozoites for 10 min at 37 °C. In these assays, EhCP112(-) trophozoites exhibited a significant decrease in the rate of

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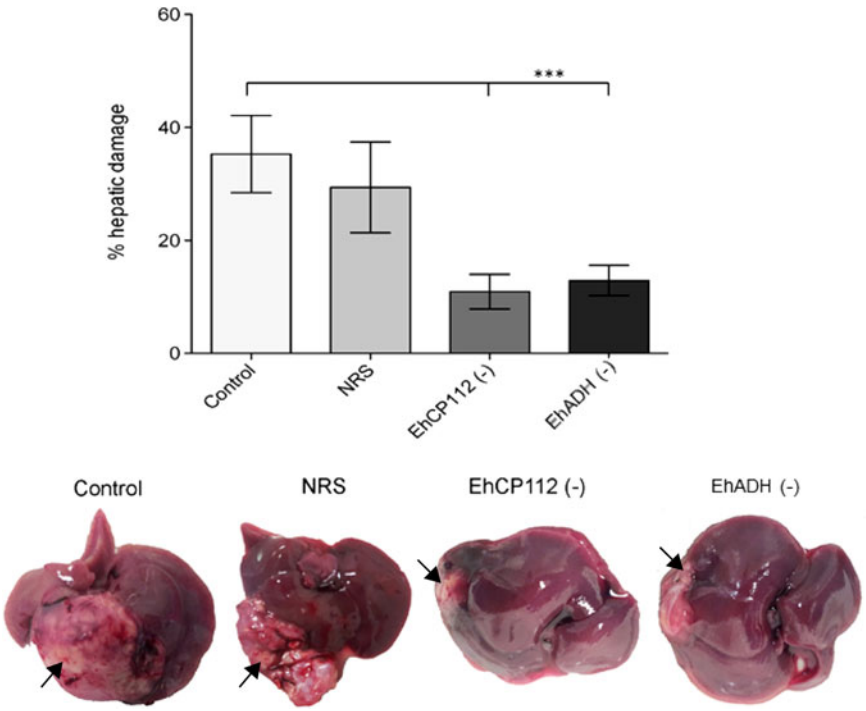

Fig. 5. *In vivo* virulence of the transfected trophozoites. Untransfected trophozoites (control) and trophozoites transfected with shRNAs for a NRS, EhCP112 and EhADH were inoculated in the liver of hamsters ($n = 6$). Seven days after challenge, animals were sacrificed under anaesthesia and liver damage was calculated as the weight of the abscesses formed divided by the weight of the complete liver before abscessed areas were removed. Representative livers of challenged and control animals are shown. Arrows indicate liver abscesses. (***) $P < 0{\cdot}005$. Abbreviations: EhADH, *Entamoeba histolytica* adhesin; EhCP112, *Entamoeba histolytica* cysteine protease complexed with EhADH; EhCPADH, Entamoeba histolytica complex formed by EhADH and EhCP112; NRS, non-related sequence; shRNAs, short-hairpin RNA.

phagocytosis (39·7% efficiency) (Fig. 4C). On the other hand, EhADH(-) trophozoites displayed a moderate, but significant, decrease in efficiency (69·8% of controls) (Fig. 4C).

Finally, in order to investigate whether knockdown of EhCP112 and EhADH affected the adherence capacity of trophozoites, EhCP112(-) and EhADH(-) strains were incubated with hRBC for 10 min at 4 °C followed by quantification of adherent erythrocytes. In these assays, the adherence capacity of EhCP112(-) trophozoites was only 22·2% compared with the control (Fig. 4D), whereas that of EhADH(-) trophozoites decreased to 32·5% (Fig. 4D).

In all these assays, trophozoites containing the shRNA for NRS did not show significant differences with respect to control trophozoites (Fig. 4A–D).

Knockdown of EhCP112 and EhADH also affected the in vivo *virulence*

To analyse whether EhCP112- and EhADH-knockingdown also affected the *in vivo* virulence of trophozoites, we tested the ability of the transfected trophozoites to produce hepatic abscesses in hamsters. All parasite-injected animals developed amoebic abscesses; however, we observed a decrease in the liver damage in the animals inoculated with the downregulated trophozoites *vs* those inoculated with the wild type strain or with the NRS trophozoites. The livers of hamsters challenged with untransfected trophozoites showed 38% of liver damage (abscessed tissue), measured as described in the Material and Methods section. Similarly, trophozoites transfected with the NRS construct resulted in 32% liver damage (Fig. 5). In contrast, hamsters inoculated with EhCP112(-) and EhADH(-) trophozoites presented only 12 and 13·6% liver damage, respectively (Fig. 5). These results confirmed that EhCP112 and EhADH proteins are important for the *in vivo* virulence of *E. histolytica*, because their knockdown decreased the trophozoites capacity to produce tissue damage.

DISCUSSION

It has been shown previously that a cysteine protease and an adhesin form the EhCPADH complex, and that this complex is involved in the pathogenic mechanism of *E. histolytica* (Arroyo and Orozco, 1987; Madriz *et al.* 2004; Martinez-Lopez *et al.* 2004; Ocadiz *et al.* 2005; Betanzos *et al.* 2013). EhCP112 and EhADH have been shown to be expressed as separate proteins encoded by different genes, as well as being able to form the EhCPADH complex (Garcia-Rivera *et al.* 1999; Banuelos *et al.*

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2005). In the present study, we addressed the hypothesis that expression of the individual proteins, EhCP112 and EhADH, is co-regulated, and in turn directly influences the formation of the EhCPADH complex. It was reasonable to assume that when one of the components is downregulated it should be a diminishing in the amount of the EhCPADH complex, but it was difficult to predict the behaviour of the free fraction of the other component. Here, using a shRNA-based system to downregulate each component of the EhCPADH complex we analysed the expression of both proteins individually, as well as the complex itself, in knockingdown trophozoites. By this strategy we showed that the diminishing expression of each EhCPADH component affected negatively the formation of the complex. Interestingly, knockdown of EhCP112 resulted in a decreased expression of EhADH and, similarly, the downregulation of EhADH also produced a reduction in EhCP112 expression. These results confirmed that EhCP112 and EhADH expression is co-regulated, probably to ensure the presence of both proteins required for complex formation, and to facilitate performance of their activities in a coordinated manner.

Gene clusters containing functionally related and co-regulated genes, also known as 'operons', are common in prokaryotic genomes; they are transcribed as one polycistronic messenger RNA from a single promoter (Dandekar *et al*. 1998). Clustering of genes with related functions and subject to common regulation has also been reported for many eukaryotic organisms, including genes of *Saccharomyces cerevisiae* encoding for galactose utilization pathway (Hittinger *et al*. 2004), genes for the homeobox (Hox) transcription factors that determine the developing embryo body plan in *Drosophila* (Lewis, 1978), gene plants for enzymes from secondary metabolic pathways (Boycheva *et al*. 2014) and human genes for the major histocompatibility complex (Horton *et al*. 2004). Likewise, gene clusters encoding proteins involved in pathogen–host recognition have been described in filamentous fungi (Collemare *et al*. 2008). These data indicate that genes that have similar and/or coordinated expression, including virulence factors, are frequently clustered. In contrast to prokaryotic operons, most of eukaryotic gene clusters subject to common regulation are transcribed as independent mRNAs.

As we know, this is the first study showing an expression co-regulation of clustering genes in *E. histolytica*. Collinearity of genes coding for the EhCPADH complex could facilitate their co-expression to provide an optimal ratio of the components and better functioning of the complex. Similarly to majority of eukaryotic clustered genes, *Ehcp112* and *Ehadh* genes are transcribed by independent RNAs (Garcia-Rivera *et al*. 1999). It has been suggested that transcriptional co-regulation of clustered genes in eukaryotes could be associated with shared regulatory elements and opening/closing of chromatin areas (Gaunt, 2015). Recently was demonstrated that silencing mediated by RNA interference in *E. histolytica* generates an increase of dimethylation of lysine 27 in H3 histone (H3K27me2), a repressive histone mark, in the downregulated gene (Foda and Singh, 2015). Therefore, downregulation of *Ehcp112* or *Ehadh* genes by shRNA may prompt an enrichment of repressive chromatin that affects the transcriptional activity of the adjacent gene, suggesting that their co-regulation could be due to chromatin opening and closing. Previous studies on the *Ehcp112* and *Ehadh* gene promoters showed that DNA regions involved in transcription activation of these genes shared the Upstream Regulatory Elements 1 and 3 (URE1 and URE3, respectively) and sequences that putatively bind the CAATT/Enhancer Binding Protein (Azuara-Liceaga *et al*. 2005; Flores-Soto *et al*. 2005), insinuating that common regulatory elements could also participate in the expression co-regulation of these genes.

A major finding in this study is the confirmation that EhCP112 and EhADH play an important role in the cytopathic mechanisms of *E. histolytica*. By knockingdown either the cysteine protease or adhesin transcripts, there was little protein co-localization in the plasma membrane of trophozoites implying a clear decrease in EhCPADH complex formation. Downregulation of the complex affected the ability of trophozoites to damage target cells *in vitro* and to produce hepatic abscesses in animals, thus providing a functional linkage between these proteins and parasite-induced pathology. However, due to the finding that knockdown of one protein also diminished the expression of the other EhCPADH component, we do not know the specific role played by each protein of the complex in these pathogenic mechanisms. Probably, the decrease of EhADH is implicated in the diminishing of adherence and phagocytosis due to the participation of this polypeptide as an adhesin and as a component of the Endosomal Sorting Complexes Required for Transport, machinery involved in the formation of mutivesicular bodies in late endosomes (Martinez-Lopez *et al*. 2004; Banuelos *et al*. 2005). Due to its protease activity (Ocadiz *et al*. 2005; Ocadiz-Ruiz *et al*. 2013), it is reasonable to speculate that knockdown of EhCP112 resulted in decreased cell lysis and phagocytosis, as well as abscesses formation.

In conclusion, in this study we demonstrated that expression of EhCP112 and EhADH proteins are coordinately regulated, as evidenced by a coincidental downregulation of both individual proteins and their complex by the knockdown of a single protein. In addition, we showed that both proteins, either by themselves or as part of the EhCPADH complex, play an important role in the *in vitro* and *in vivo* virulence of *E. histolytica*.

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SUPPLEMENTARY MATERIAL

To view supplementary material for this article, please visit http://dx.doi.org/10.1017/S0031182015001470X.

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