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The ubiquitin-activating enzyme (E1) of the early-branching eukaryote *Giardia intestinalis* shows unusual proteolytic modifications and play important roles during encystation

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ABSTRACT

Giardia intestinalis is considered an early-branching eukaryote and is therefore a valuable model for studying primordial cellular processes. This work reports the characterization of the ubiquitin-activating enzyme (E1) during growth and different stages of trophozoite differentiation into cysts. We found that in *Giardia* E1 expression (both at mRNA and protein levels) is regulated during encystation. The enzyme is proteolytically processed mainly into two fragments of 68 kDa (N-terminal) and 47 kDa (C-terminal). This phenomenon has not been described for any other E1. In trophozoites, this enzyme localized at spots within the cytoplasm as detected by using polyclonal antibodies against either E1 N- or C-terminal fragments. This pattern changed during encystation into a diffuse localization throughout the cytoplasm of encysting cells. E1 localizes in mature cysts at cytoplasmic spots and in the cyst wall. Our antisense silencing experiments suggested that E1 is an essential gene for parasite viability. On the other hand, E1 over-expression greatly increased the encystation rate, indicating a relationship between E1 and *Giardia* differentiation.

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1. Introduction

Giardia intestinalis is a pathogenic microorganism inhabiting the upper small intestine of humans and many other vertebrates, and is one of the most frequent diarrhoea-causing parasites worldwide (Adam, 2001). *G. intestinalis* is a binucleated, flagellated protozoan which is considered one of the earliest branching eukaryotes (Svard et al., 2003). The complete *G. intestinalis* genome has been reported, showing that this parasite have less complex molecular machineries than other eukaryotes (Morrison et al., 2007). Additionally *G. intestinalis* lacks some of the typical organelles of higher eukaryotic cells, such as mitochondria, peroxisomes and a classical Golgi apparatus (Adam, 2001). *Giardia* possesses an interesting life cycle that may be a primitive adaptation to different environmental conditions, permitting *Giardia* to survive both within and outside hosts, alternating between its two stages: trophozoites and cysts (Lujan et al., 1998). The trophozoite is the motile and vegetative stage, which lives and colonizes the upper small intestine by attaching to the epithelial cells. The quadrinucleated cyst is the transmissible stage and is extremely resistant to harsh environmental conditions due to the presence of a protective cell wall. The life cycle begins when cysts are ingested by the host from contaminated water, food, or interpersonal contact. The cysts sense the low pH in the stomach and start a process called excystation, which is finally completed in the upper small intestine where the trophozoites rapidly emerge and proliferate. Some trophozoites are forced down with the intestinal flow and are induced to encyst when confronting low cholesterol concentration in the lower parts of the small intestine. Cysts mature in the large intestine and are then released with the feces (Lujan et al., 1998; Adam, 2001). *Giardia*'s encystations involves important molecular and cellular processes (DNA replication, nuclear division), turnover of proteins, and a special regulation of synthesis, sorting and transport of cyst wall components (Carranza and Lujan, 2010).

The ubiquitin–proteasome system (Ub–P) is the main cellular machinery for protein turnover in eukaryotic cells (Glickman and Ciechanover, 2002). Degradation by Ub–P involves two steps. Target proteins are selectively, specifically, and covalently attached to ubiquitin (Ub) chains, followed by Ub-tagged proteins becoming unfolded and degraded by the 26S proteasome multiprotein complex. The Ub conjugation to proteins (or ubiquitination) is an enzymatic cascade consisting of three sequential steps, resulting in

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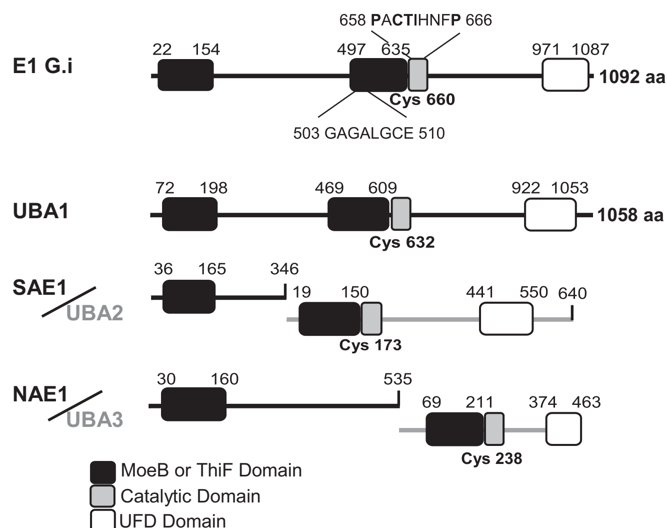

Fig. 1. E1 domain structure. Pfam (www.pfam.sanger.ac.uk) and SMART (http://smart.embl-heidelberg.de) softwares were used for identifying the domains present in *Giardia* E1 sequence A8BBP6. They were compared with *Homo sapiens* sequences for ubiquitin-activating enzyme UBA1 (P22314), heterodimeric SUMO-activating enzyme SAE1/UBA2 (Q9UBE0 and Q9UBT2 respectively) and heterodimeric NEED8-activating enzyme NAE1/UBA3 (Q13564 and Q8TBC4 respectively). The numbers indicate residue positions. MoeB or ThiF repeat domains are shown in black, catalytic domain in gray and the ubiquitin folding domain (UFD) in white. The position of the conserved cystein residue involved in covalent thioester linkage with ubiquitin is shown with bold black numbers. Conserved ATP-binding domain motif (GXGXXGCE) and the putative active site (PXCTXXXXP) sequence are shown in *Giardia* E1 sequence.

Ub attachment to the ε-amino group of a lysine (Lys) residue in the target protein. Conjugation begins with ATP-dependent Ub activation by the ubiquitin-activating enzyme (E1). The E1 enzyme loads two Ub molecules at two different sites; one Ub is covalently linked by its glycine 76 carboxyl group to a cysteine at the E1 catalytic site by a thioester linkage, and a second Ub is non-covalently associated as an adenylate at the E1 adenylation site. The Ub in the active E1 site is transferred to the ubiquitin-conjugating enzyme (E2). The E2 transiently carries the activated Ub molecule as a thiol ester and this activated Ub is then transferred to the target protein bound to one of the multiple ubiquitin-ligases (E3). Several Ub molecules are then added to the Lys residues in the Ub bound to the substrate protein and these chains are recognized by the 26S proteasome (Pickart, 2001; Glickman and Ciechanover, 2002). The Ub–P system plays important roles in many cell functions, such as cell cycle progression, antigenic presentation, and inflammatory response. Defects in this system in humans are related to several diseases (King et al., 1996; Pickart, 2001). The Ub tagged protein may take different pathways to degradation, depending of the kind of Ub-chains or the amount of Ub molecules attached to it. Ub-chains having at least four Lys48-linked Ub subunits generally trigger tagged protein degradation by the proteasome. Lys63-linked Ub-chains have functions in DNA repair and endocytosis. Monoubiquitination also plays important roles in endocytosis, membrane trafficking, DNA repair and histone regulation. Multiubiquitination (adding multiple monomeric Ub) is involved in endocytosis (Woelk et al., 2007).

The E1 enzyme is the apex for downstream enzymatic cascades and signaling pathways mediated by Ub and Ub-like proteins (Ubls) (Pickart, 2001). Studying characteristics of E1 and its catalytic functions may throw light to the role of ubiquitination in cell development. All known eukaryotic E1 are monomeric 110–120 kDa proteins, although the E1 of either SUMO or NEDD8 (Ub-like proteins – Ubl) present heterodimer complexes and all have three common domains (Fig. 1). They have one domain with two MoeB or ThiF adenylation sequence repeats in the N-terminal for Ub E1. One MoeB or ThiF repeat binds ATP and Ub as an adenylate and the other provides structural stability (Walden et al., 2003; Lois and Lima, 2005; Lee and Schindelin, 2008). These two MoeB or ThiF repeats are separated in SUMO and NEDD8 E1 (one in each subunit). The second one is the catalytic domain having the cysteine residue involved in covalent thioester linkage with Ub. The third domain is the ubiquitin folding domain (UFD) at the carboxy-terminal end of E1 that binds to the E2 (Komatsu et al., 2001; Lee and Schindelin, 2008; Schulman and Harper, 2009).

A characterization of *G. intestinalis* E1 is reported here. It is shown that the enzyme is time-specifically expressed during encystation and that it undergoes an unique post-translational processing generating two fragments of 68 kDa (N-terminal) and 47 kDa (C-terminal). It was found, using polyclonal antibodies against E1 N- or C-terminal regions, that E1 localizes in the trophozoites in a precise pattern bound to small granules or vesicles. The lethality of RNA antisense silencing of *Giardia* E1 indicates that E1 is essential. On the other hand, the over-expression of E1 greatly increases the encystation rate, thereby demonstrating a direct relationship between E1 and *G. intestinalis* differentiation. These results show that *Giardia* E1 has a different behavior to that found in other eukaryotes and this could be a clue for the evolution of these enzymes. This is the first report of a direct involvement of E1 (and maybe of the ubiquitination pathway) in the differentiation of this early-branching eukaryote.

2. Materials and methods

2.1. G. intestinalis culture, encystation and transfection

G. intestinalis isolate WB/9B10 trophozoites (Carranza et al., 2002) were grown in TYI-S-33 medium supplemented with 0.5 mg/ml bovine bile and 10% bovine serum (Keister, 1983). Trophozoites were encysted *in vitro* according to the procedure described by Kane et al. (1991). The transfection method was that described by Yee and Nash (1995). Plasmid-carrying trophozoites were selected with puromycin.

2.2. RNA isolation, cDNA synthesis and quantitative real-time PCR

Total RNA was isolated from proliferating trophozoites and encysting cells using TRIZOL reagent (Invitrogen). The samples were evaluated by PCR to control for any DNA contamination. Five micrograms of total RNA were used for synthesizing cDNA with M-MLV reverse transcriptase (Promega) and poly-T primer in a 25 μl volume reaction. Five microliters of cDNA were used as template in the quantitative PCR reactions with Quantitect SYBR Green PCR (Qiagen) using a Chromo 4 real-time PCR detector (BioRad). Regarding quantification, the calibration curve was drawn (in triplicate) with the pGEM-T-GE1NT plasmid that contains the E1 DNA fragment. Gl-NTE1-F 5′-GAA TTC ACC CTA CGT CAA CAT CAT-3′ and Gl-NTE1-R 5′-GTC GAC ATT GTC AAC CAC CTC AAC-3′ oligonucleotides were used to amplify a 145 bp segment from the E1 gene (UniProt A8BBP6). RT-PCR for checking E1 mRNA integrity was done using the cDNA template with Gl-NTE1-F 5′-GAA TTC ACC CTA CGT CAA CAT CAT-3′ and Gl-CTE1-R 5′-GTC GAC CCT TGA ACT GTT ACG GTT-3′ oligonucleotides amplifying a 2898 bp segment from the E1 gene (158–3056 bp). This fragment contained the two regions used for generating the recombinant proteins (see below). The products were analyzed by electrophoresis on 0.8% agarose gels in TBE 0.5× and stained with ethidium bromide.

2.3. Expression and purification of recombinant E1 proteins

Two recombinant proteins were used as antigens for producing polyclonal antibodies in mice. Two E1 gene fragment were

amplified by PCR: the N-terminal region (158–303 bp) with G-NTE1-F 5′-GAA TTC ACC CTA CGT CAA CAT CAT-3′ and Gl-NTE1-R 5′-GTC GAC ATT GTC AAC CAC CTC AAC-3′ oligonucleotides and the C-terminal region (2744–3056 bp) with G-CTE1-F 5′-GAA TTC ATG ACA TCC CGA CTA TTG-3′ and G-CTE1-R 5′-GTC GAC CCT TGA ACT GTT ACG GTT-3′ primers. Oligonucleotides were designed according to the *G. intestinalis* A8BBP6 sequence in the UniProt database. Restriction sites were added for EcoRI and SalI (underlined). PCR was performed with AccuPrime Pfx DNA polymerase (Invitrogen). The products were visualized by horizontal electrophoresis on 1.5% agarose gels in TBE 0.5× and ethidium bromide staining. PCR products were purified using a Promega Wizard Kit PCR Preps DNA purification system. The purified products were ligated to the pGEM-T (Promega) vector and transfected by electroporation of *Escherichia coli* JM109 competent cells; this approach led to pGEM-T-GNET1 and pGEM-T-GCTE1 clones. Plasmid DNA was purified and inserts were released by double digestion with EcoRI and SalI. Digestion products were purified with a Promega Magic Clean-Up System and ligated to pThioHis expression vector (Invitrogen). These vectors were transfected by electroporation of *E. coli* JM109 competent cells. Recombinant protein (rNTE1 and rCTE1) synthesis was induced by incubation with IPTG (100 μM) for 4 h. The bacterial lysate was obtained by sonication and thermal shock and His-Patch thioredoxin tagged recombinant proteins rNTE1 (18 kDa) and rCTE1 (25 kDa) were partially purified by nickel affinity chromatography using the ProBond resin system (Invitrogen). rNTE1 and rCTE1 were completely purified by extracting proteins from the SDS-PAGE polyacrylamide gel (Scheer and Ryan, 2001).

2.4. Anti-E1 antibodies production

Pure recombinant proteins were used as antigens for producing polyclonal antibodies in six-week-old female BALB/c mice. Four 30 μg antigen subcutaneous inoculations were made (one per week); the mice were euthanized and sera (anti-gNTE1 and anti-gCTE1) were obtained by centrifuging the coagulated blood at 800 × *g* for 30 min.

2.5. Immunoblot analysis

Protein was extracted by suspending the cell pellet in extraction buffer (30 mM Tris, pH 7.5, 50 mM NaCl, 3 mM $MgCl_2$, 10% glycerol, 10 mM 2-mercaptoethanol, 0.5% NP40 and P8340-Sigma protease inhibitor cocktail) and incubated for 1 h at 4 °C with shaking. The suspension was then centrifuged at 8000 × *g* for 30 min at 4 °C. The supernatant (cytoplasmic extract) was carefully collected and the pellet conserved to produce the nuclear protein extract. Nuclear extraction was performed by suspending the pellet in nuclear extraction buffer (30 mM Tris, pH 7.5, 400 mM NaCl, 3 mM $MgCl_2$, 10% glycerol, 10 mM 2-mercaptoethanol, 0.5% NP40 and protease inhibitor cocktail) and incubated for 1 h at 4 °C with shaking. The suspension was then centrifuged at 8000 × *g* for 30 min at 4 °C, the supernatant (nuclear extract) was carefully collected and protein concentration quantified by Bradford's method. Aliquots were stored at −20 °C. The cysts had to be crushed in liquid N_2 (to disrupt the cyst wall) before extraction. Protein samples were treated with sample buffer (50 mM Tris–HCl, pH 6.8, 100 mM DTT, 2% SDS, 0.1% bromophenol blue and 10% glycerol) and analyzed by SDS-PAGE on polyacrylamide gel gradients (5–20%). The gel was blotted onto a PVDF-cellulose membrane (200 mA for 2 h in 100 mM Tris, 192 mM glycine, 1% SDS, 10% methanol, pH 8.3). The membrane was blocked with TBST-milk (20 mM Tris–HCl, pH 7.5, 150 mM NaCl, 0.1% (v/v) Tween 20 and 5% milk) for 1 h at room temperature with constant agitation. The filter was then incubated for 1 h with the primary antibody (anti-gNET1 or anti-gCTE1) diluted 1:500 in TBST. Regarding the detection of the HA epitope tag an anti-HA mAb (Sigma) was used at 1:1000 dilution. The membrane was washed three times with TBST-milk following incubation with biotinylated mouse anti-IgG (Sigma) at 1:3000 in TBST and washed again three times in TBST-milk. The membrane was then incubated for 30 min with 1:3000 streptavidin-alkaline phosphatase conjugate (Promega) in TBST, washed three times with TBST and finally incubated with the NBT-BCIP substrate (Promega).

2.6. Immunofluorescence microscopy

Cells were fixed with 4% p-formaldehyde and then permeabilized for 1 h at room temperature in phosphate-buffered saline, 0.1% Triton X-100, 10% goat serum. Slides were incubated for 1 h at 37 °C with the antibodies diluted in phosphate-buffered saline (1:500 anti-gNTE1 and anti-gCTE1, 1:1000 mAb anti-HA). Cells were then washed three times with PBS and incubated with the anti-mouse secondary antibody labeled with FITC (Sigma, 1:1000 final dilution). The anti-CWP1-TAMRA antibody (Waterborne Inc.) was used for detecting the CWP1 protein (1 h at 37 °C). Controls included the omission of primary antibodies. Slides were viewed on a Leica IRME fluorescence microscope and the images were captured with a Hamamatsu ORCA ER II camera and processed with Leica QFluoro software (Leica Microsystems).

2.7. E1 antisense silencing

A E1 gene fragment was inserted in the pTubHApacCT vector (Touz et al., 2003) inversely to generate an antisense vector. For amplifying an E1 gene 600 bp fragment by PCR we used the sense oligonucleotide UbiE1AS-F 5′-GCG GAT ATC ATG AAT CGA GAC TAT TCT CGC AC-3′ (containing an EcoRV restriction site), and antisense oligonucleotide UbiE1AS-R 5′-CAT GCC ATG GGA AAA AGC GCC AGT CAC G-3′ (containing a NcoI restriction site) (restriction sites are underlined). A PCR product was generated, restricted and purified as described before. The product was cloned into the pTubHApacCT vector to generate pTubHApacCT-E1as antisense vector. Trophozoites were transfected by electroporation and selected with puromycin.

2.8. E1 over-expression

The complete E1 coding region was cloned into the pTubHApacNT and pTubHApacCT independently. Cloning into the pTubHApacNT with oligonucleotides NT-UbiE1-F 5′-CGC GGA TCC ATG AAT CGA GAC TAT TCT CGC AC-3′ and NT-UbiE1-R 5′-ATA AGA ATG CGG CCG CCT AAT TTA CTA GGC AAA GTT TGG G-3′ (BamHI and NotI restriction sites, respectively), and cloning into the pTubHApacCT vector with oligonucleotides CT-UbiE1-F 5′-CAT GCC ATG GAT GAA TCG AGA CTA TTC TCG CAC-3′ and CT-UbiE1-R 5′-GCG CGA TAT CAT TTA CTA GGC AAA GTT TGG G-3′ (NcoI and EcoRV restriction sites, respectively). PCR, cutting, purification and cloning was done as described above. These vectors allow the constitutive and stable expression of genes in *Giardia* trophozoites and the protein is generated with an HA tag (HA-N-terminal tag with pTubHApacNT and HA-C-terminal with pTubHApacCT) (Touz et al., 2003). Trophozoites were transfected by electroporation and selected with puromycin. Trophozoites were analyzed by immunofluorescence and immunoblotting.

2.9. In vitro ubiquitination assay

The *G. intestinalis* trophozoite (wild-type or transfected cells) NP40 protein extract was used in the *in vitro* ubiquitination assay as a source of ubiquitin-activating enzyme (E1), ubiquitin-conjugating enzymes (E2), ubiquitin-ligases (E3) and substrates. The reaction mix contained 250 ng His_6-ubiquitin

(Sigma), 0.5 U/μl phosphocreatine kinase (Sigma), 10 mM creatine phosphate (Sigma), 1.0 mM ATP (Sigma), 2.5 mM $MgCl_2$ (Sigma), 2 μM ubiquitin-aldehyde (Boston Biochem), 20 μM MG132 (Boston Biochem), 10 mM Tris–HCl pH 7.5 and 50 μg protein extract. Final reaction volume was 15 μl. The reaction was incubated at 25 °C for 30 min; and stopped by adding 5 μl of 4× sample buffer and incubating the mixture for 10 min at 95 °C. The ubiquitinated proteins were detected by immunoblot with antibody anti-histidine (Amersham).

3. Results

3.1. The G. intestinalis E1 primary sequence possesses all the E1 characteristic domains

The *G. intestinalis e1* gene (UniProt A8BBP6) has been previously identified by us using bioinformatics tools (Gallego et al., 2007). Analysis of this sequence (here) showed that it encodes a 1092 amino acid E1 protein (121 kDa). It has the E1 family's three characteristic domains (Fig. 1), two MoeB or ThiF repeats, a catalytic domain and a C-terminal UFD site that binds to E2; these domains are located in regions similar to those of human E1 (UBA1) (Lois and Lima, 2005; Lee and Schindelin, 2008). The *Giardia* E1 sequence contains two important consensus motifs which are conserved amongst all E1 enzymes: a highly conserved ATP-binding domain motif GXGXXGCE (GAGALGCE, amino acids 503–510) and a putative active site PXCTXXXXP (PACTIHNFP, amino acids 658–666) with Cys660 forming the thioester linkage with Ub (McGrath et al., 1991; Hatfield and Vierstra, 1992).

3.2. E1 undergoes a post-translational proteolytic process

The full length E1 protein was over-expressed in trophozoites having an HA tag at the N-terminal or C-terminal end. Trophozoites were transfected with pTubHApacNT-E1 (HA-N-terminal tag) and pTubHApacCT-E1 vector (HA-C-terminal tag), independently. Cells were selected with puromycin and evaluated by immunoblotting and immunofluorescence microscopy with antibodies against the HA tag and antibodies against the E1 N-terminal (anti-gNTE1) or C-terminal regions (anti-gCTE1). These anti-E1 antibodies were produced in mice using N-terminal or C-terminal recombinant proteins as antigens. The polyclonal sera produced (anti-gNTE1 and anti-gCTE1) showed an excellent response against the recombinant proteins and did not cross-react with other bacterial proteins (Fig. 2A). Fig. 2B shows immunofluorescence analysis of trophozoites using anti-gNTE1 (anti-gCTE1 had a similar pattern, data not shown). E1 was located in the trophozoite cytoplasm in several small spots, probably associated with membranes or protein complexes and was not free in the cytoplasm. E1 showed the same pattern in transfected cells as in wild type cells, but with a more intense signal (Fig. 2B). A more defined localization in membranes or very close to them in transfected cells showed that E1 could be associated with the recognition of signals through the membrane or to membrane proteins. The less defined membrane signal in wild type cells may be due to their lower concentration or to a fast turnover, which could only be detected during E1 over-expression. No signals were detected in immunofluorescence assays with mAb anti-HA (Fig. 2B). This suggests that E1 was processed in its N- and C-terminal end segments, losing the HA terminal tags. The pTubHApac-NT/CT vector has been used by us several times to over-express different kind of proteins in *G. intestinalis* (Touz et al., 2003; Gottig et al., 2006; Elias et al., 2008), this is the first time that we observe this event. The absence of the tag was the first evidence of a proteolytic E1 processing. Immunoblot analysis with anti-E1 antibodies showed an unusual pattern compared to other E1s (Fig. 2C). A 68 kDa (E1-68) and a 40 kDa fragments (E1-40) were detected with anti-gNTE1 and anti-gCTE1, respectively. These proteins were only revealed in wild type cells when 100 μg or more protein was used in immunoblot (Fig. 4B).

An *in vitro* ubiquitination assay was performed to define E1 activity in wild type and transfected cells (Fig. 2D). The assay consisted of generating ubiquitin–protein conjugates (His-tagged) using the protein extracts as a source for the ubiquitination system (E1, E2s, E3s and protein substrates). The reaction mix had His_6-ubiquitin, proteasome, ubiquitin-hydrolase inhibitors, and an ATP-regenerating system. The results showed that E1 over-expression produced more abundant ubiquitinated products than the wild type. This result demonstrated that post-translational E1 processing did not affect its catalytic activity and is also an evidence for the functionality of the cloned gene.

3.3. E1 expression is regulated during encystation

Real time RT-PCR analysis (Fig. 3A) was performed to determine E1 mRNA expression during encystation. Trophozoites were induced *in vitro* to encyst and cell samples were collected at different periods (0, 6, 12, 24 and 48 h). Total RNA was extracted and used for cDNA synthesis before real-time PCR reaction. E1 mRNA levels increased during the first 12 h of encystation when the highest expression level was obtained, being three times greater than in proliferating trophozoites (Fig. 3A). E1 transcripts then decreased and reached the lowest level during the cyst stage (48 h), approximately 1/3 compared with that of trophozoites. This result showed that E1 transcription was regulated during encystation. E1 mRNA integrity was evaluated by RT-PCR using oligonucleotides that amplify a 2898 nucleotide fragment (this fragment contained the two regions used for generating recombinant proteins), almost the complete predicted E1 mRNA (3279 nt). The product was amplified and the results revealed the presence of a complete E1 transcript; the two regions used for recombinant protein generation were present in this mRNA so they were both transcribed in the same mRNA (Fig. 3B).

Protein expression analysis was performed to determine the E1 behavior during the encystation and whether the proteolytic process occurred to the endogen protein in wild cells. Trophozoite cytoplasm and nuclear protein extracts were used in immunoblots for detecting endogenous E1. Only a slight signal was detected, even when using a high amount of protein (100 μg or more). The immunoblot results (Fig. 3C and D) showed similar behavior to that observed in transfected cells (Fig. 2C). The anti-gNTE1 immunoblot showed three signals, a protein of around 114 kDa (E1-114), another strong 68 kDa signal (E1-68), and a 90 kDa protein (E1-90). We propose that the 114 kDa protein (E1-114) may represent the complete E1 protein (121 kDa predicted molecular mass). This agrees with the over-expression results that suggested specific proteolytic N- and C-terminal processes. Anti-gCTE1 immunoblotting showed two bands of around 47 (E1-47) and 40 kDa (E1-40). These results again demonstrated E1 processing. The E1-68 and E1-47 species were the strongest bands using respectively anti-gNTE1 and anti-gCTE1 antibodies. Only the E1-68 and E1-40 species were detected in the transfected cells, suggesting that over-expression increased this specific proteolytic process. The presence of extra bands, maybe by proteolytic processing, was observed previously in other *G.intestinalis* protein; Touz et al. (2008) detected several lower molecular weight extra bands in the immunodetection of Arginine deaminase (ADI). It is important to stress that we included proteases inhibitors to prepare the lysates, therefore we suggest that this extra bands are produced by a specific proteolityc event on E1. In summary, we propose that E1 was transcribed (Fig. 3B) and translated as the full length form (E1-121) and then quickly processed to generate E1-114 and then the two main 68 (E1-68)

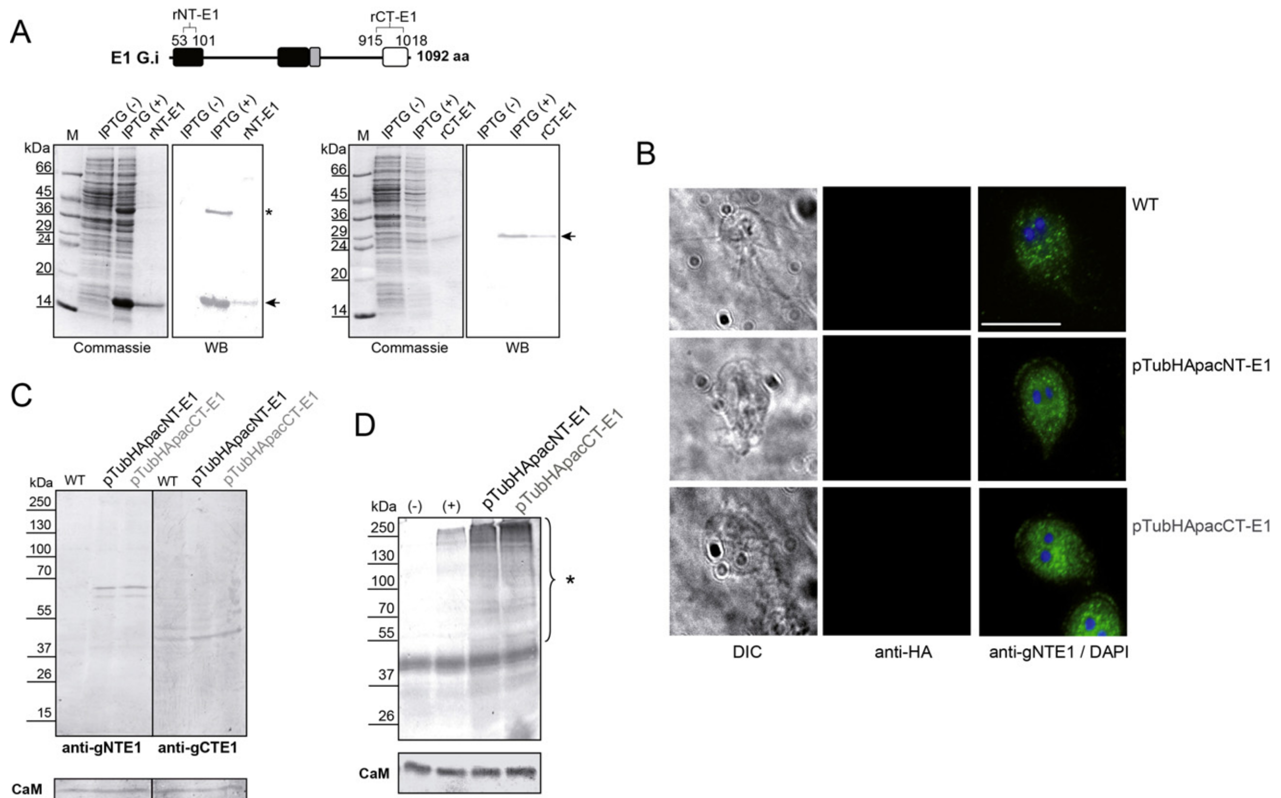

Fig. 2. E1 overexpression in transfected cells. (A) Evaluation of anti-gNTE1 and anti-gCTE1 antibodies against E1 recombinant proteins (rNT-E1 and rCT-E1). Localization of portions of the protein E1 against which the antibodies were raised is shown in the top scheme. Thirty μg of bacteria lysate (IPTG not induced (−) and induced (+)) and two hundred ng of each recombinant purified protein were subjected to SDS-PAGE and stained with Coomassie blue or blotted onto PVDF membranes. The left panel shows the Coomassie blue staining and the right panel (WB) shows the immunobloting with anti-E1 antibodies. The blot was probed with anti-gNTE1 1:5000 (1) and anti-gCTE1 1:5000 (2). The asterisk shows an rNT-E1 dimer, and the arrows indicate the recombinant proteins position. (B) Localization of endogenus and overexpressed E1 in wild (WT) and transfected (pTubHApacNT-E1 and pTubHApacCT-E1) trophozoites. The complete *e1* was cloned in pTubHApacNT and pTubHApacCT expression vectors as described in Section 2. Wild type and transfected trophozoites were collected, fixed with p-formaldehyde and subjected to immunofluorescence analysis with anti-E1 antibodies (1:500), monoclonal anti-HA antibody (1:1000), and DAPI staining. Scale bar is 10 μM. (C) E1 immunoblot detection in wild type and transfected (pTubHApacNT-E1 and pTubHApacCT-E1) trophozoites. Thirty μg of lysate were subjected to SDS-PAGE and immunoblot using anti-E1 antibodies. CaM (calmodulin) was used as load control and detected with anti-gCaM 1:1000. (D) Ubiquitination *in vitro* reaction. Fifty ug of protein extract from wild type or transfected trophozoites (pTubHApacNT-E1 and pTubHApacCT-E1) were used in the *in vitro* ubiquitination as described in Section 2. Ubiquitinated proteins were detected by immunoblot with anti-His (1:5000). (−) shows the protein extract from wild type trophozoites (time zero).and (+) the protein extract from wild type trophozoites after 30 min incubation. Reactions with protein extract of transfected trophozoites are indicated as pTubHApacNT-E1 and pTubHApacCT-E1, respectively. CaM (calmodulin) was used as load control and detected with anti-gCaM 1:1000. The asterisk labeled area corresponds to His_6-ubiquitin products.

and 47 kDa (E1-47) fragments. On the other hand, we suggest that the E1-68 and E1-47 forms underwent additional post-translational modifications to generate E1-90 and E1-40 kDa forms, respectively.

E1 protein behavior was similar to that of the mRNA during encystation. The most abundant forms during differentiation into cysts were E1-68 and E1-47, the levels of which increased during the first 12 h of induction and then decreased (Fig. 3D). The complete E1-114 was detected in non-encysting trophozoites (faint signal) and in early encysting cells (6 and 12 h) where E1-68 had the highest expression. These results showed that E1 was developmentally regulated during encystation and reached its highest level 12 h after the process had been initiated.

3.4. E1 localization during encystation

Non-encysting and encysting trophozoites were collected, fixed, and subjected to immunofluorescence microscopy. Fig. 4 shows E1 location in proliferating trophozoites, encysting cells, and cysts. Similar localization patterns were found with both antibodies (anti-gNTE1 and anti-gCTE1). E1 was located in non-encysting trophozoite cytoplasm as a punctuate pattern. That pattern likely indicated that E1 was associated with membranes or highly structured protein complexes. The specific pattern in encysting cells changed to a more diffuse localization, showing an important distribution change, which might be linked to regulated expression of E1. On the other hand, E1 presented both a diffuse and punctuate pattern inside mature cysts and, interestingly, it was also detected in the cyst wall, or very close to it, suggesting cyst wall matrix association with ubiquitination. E1 could be important during either the generation of this structure because it is very close to the extracellular environment. Further studies are necessary to provide more evidence about the relationship between E1 and the cyst matrix and about its role during cyst wall formation.

3.5. E1 is essential for trophozoites and is implicated in encystation

E1 expression was down regulated by RNA antisense and up regulated by over-expression to determine its role and its functionality in *Giardia* development and differentiation. Transfection of trophozoites with pTubHApacCT-E1as carrying an E1 500 bp antisense fragment was used to silence the enzyme. No viable clones

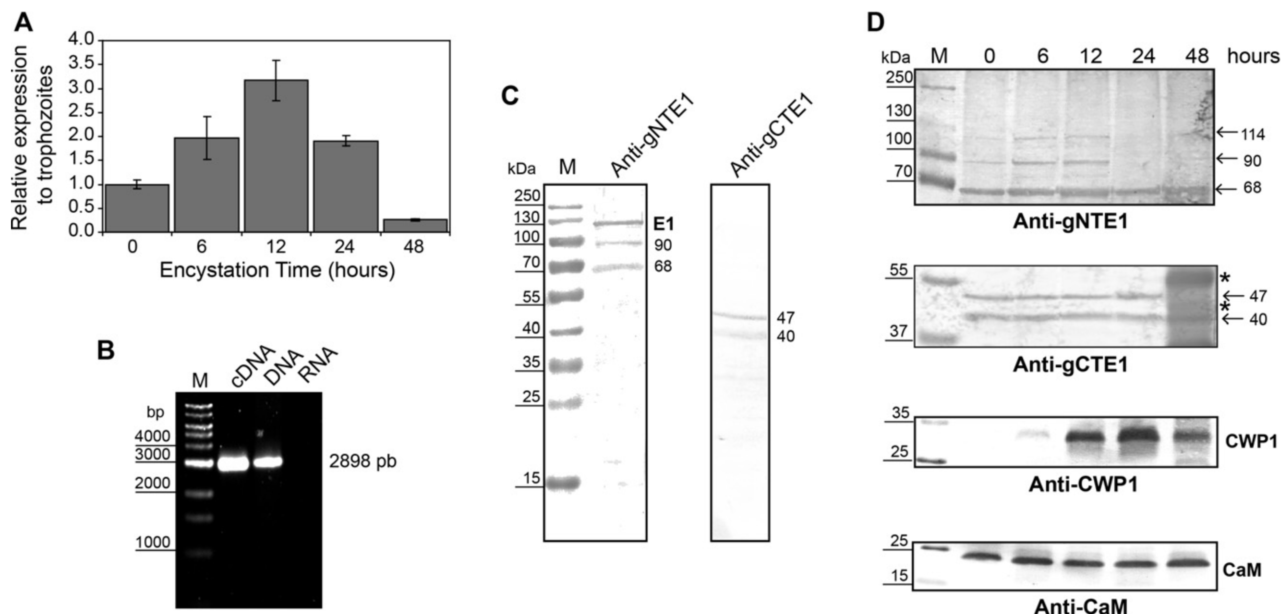

Fig. 3. E1 expression during encystation. (A) Quantitative real-time PCR of *e1* gene expression. Total RNA was isolated from trophozoites and during encystation using TRIZOL reagent. Five ug of RNA were used to synthesize the cDNA. Five microliters of cDNA were used as template in quantitative PCR reactions; the calibration curve was done (in triplicate) with pGEM-T-GE1NT plasmid containing the E1 DNA fragment. Changes in mRNA expression are shown as the ratio of transcript levels in the different encysting cells relative to trophozoites. (B) E1 mRNA integrity. RT-PCR for checking E1 mRNA integrity was done using the cDNA template with Gl-NTE1-S and Gl-CTE1-AS primers amplifying a 2898 bp segment of the *e1* gene (this fragment contained the two regions used for generating the recombinant proteins, see below). The lanes were marked as follows: M. 1 Kb marker. cDNA: PCR with 5 μl cDNA as template. DNA: PCR with 100 ng DNA as template (positive control). RNA: PCR with 200 ng RNA as template (control of RNA purity). (C) Immunoblot detection of E1 in trophozoites. Trophozoite lysates (100 μg) were subjected to SDS-PAGE and immunoblotted with anti-E1 antibodies. Left panel: immunoblot with anti-gNTE1; right panel: immunoblot with anti-gCTE1. (D). Immunoblot detection of E1 during encystation. *G. intestinalis* trophozoites were induced to encyst and cells were collected at different times during the process. Representative lysates were obtained and subjected to SDS-PAGE and immunobloting. The immunoblots were performed using anti-NTE1 (1:500) and anti-CTE1 (1:500). The arrows show the proteins detected: 114, 90 and 68 kDa with anti-gNTE1; 47 and 40 kDa with Anti-gCTE1. (*) indicate the secondary antibody's non-specific signals. Immunoblot detection of CWP1 (26 kDa) was done as encystation marker (anti-CWP1). Immunoblot detection of Calmodulin (CaM, 17 kDa) was done as load control (anti-CaM).

could be obtained in three independent experiments, while positive transfection controls (the empty vector pTubHApacCT) worked fine. This result suggested that *e1* is an essential gene in *G. intestinalis*. *In vitro* encystation analysis was performed with both wild type and transfected trophozoites to determine whether E1 has a role in encystation (Fig. 5). E1 overexpression greatly increased the encystation rate, 5- to 6-fold more than that of wild-type cells. This is first but strong evidence that E1 and ubiquitination play a role in *G. intestinalis* trophozoite differentiation into cysts.

4. Discussion

Ubiquitination is an important eukaryotic process that defines the fate of proteins and their functionality. It has multiple roles in cell survival, differentiation and development (Glickman and Ciechanover, 2002). Ubiquitination is usually known as a protein degradation signal, but many examples have shown its role as an important signal, being regulated by E3-ligase/deubiquitinating enzymes similary to that occuring by kinases/phosphatases during

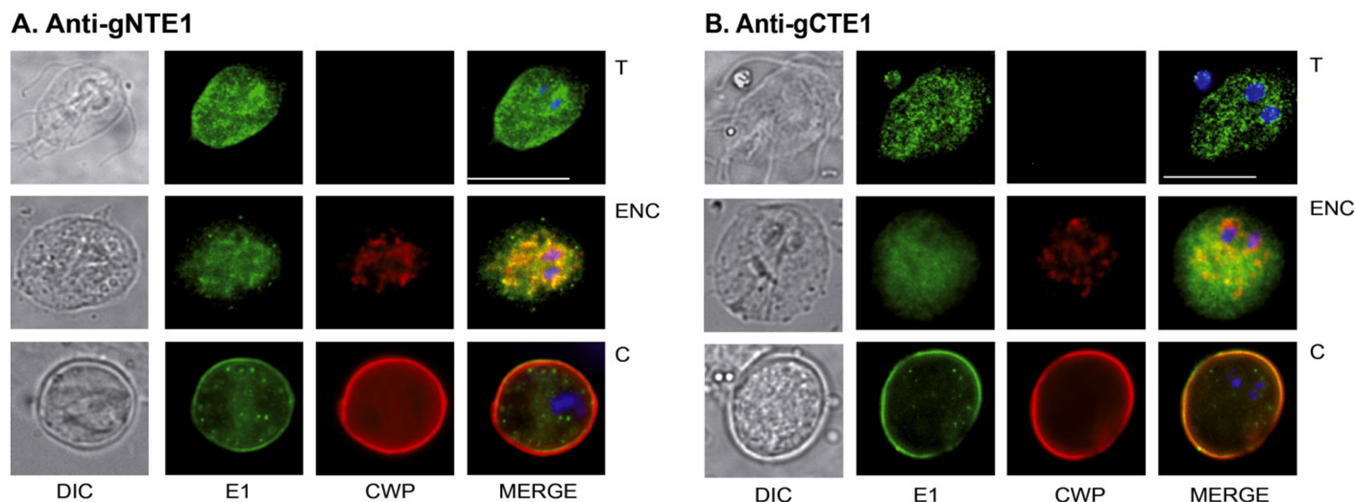

Fig. 4. E1 localization during encystation. Non-encysting and encysting trophozoites were collected, fixed with p-formaldehyde, and used for immunofluorescence analysis with anti-E1, anti-CWP1-TAMRA antibodies (Waterborne Inc.) to detect CWP1 protein, and DAPI staining for nuclei. (A) Detection with anti-gNTE1 antibody. (B) Detection with anti-gCTE1 antibody. The different stages are indicated as follows: (T) trophozoites, (ENC) encysting cells and (C) cyst. Scale bar is 10 μM. DIC is differential interference contrast. ESVs: encystation-specific vesicles.

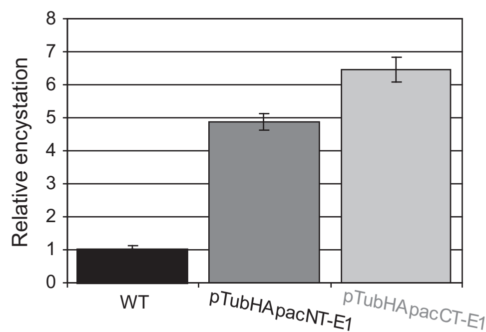

Fig. 5. Effect of E1 overexpression on *in vitro* encystation. Two thousand wild type, pTubHApacNT-E1 and pTubHApacCT-E1 transfected trophozoites were induced to encyst. The number of cysts was counted and the percentage of cyst formation was determined. Changes in encystation are shown relative to the wild type cells percentage of cyst formation. The experiment was done in triplicate.

phosphorylation/dephosphorylation (Woelk et al., 2007). Ubiquitin is present in eukaryotes but not in prokaryotes. Ubiquitination consists of an enzymatic cascade that begins with ubiquitin activation by the E1 ubiquitin-activating enzyme (Pickart, 2001; Glickman and Ciechanover, 2002). E1 enzymes are thus considered the apex for downstream enzymatic cascades and ubiquitin-mediated signaling pathways. E1 manipulation leads to defining ubiquitination's role in different cells. An E1-deficient strain dramatically reduces Ub conjugation in yeast, producing cell cycle arrest (Ghaboosi and Deshaies, 2007); E1 has been shown to have several functions during different development stages in *Caenorhabditis elegans* and *Drosophila melanogaster* (Kulkarni and Smith, 2008; Lee et al., 2008). This work focused on studying *G. intestinalis* E1 for ascertaining the relevance of ubiquitination in this primitive eukaryote's development and to explore ubiquitination's primordial roles in the eukaryotic linage.

The results showed that E1 was an essential gene for *G. intestinalis* survival and differentiation. E1 enzymes have characteristic domains that are important for their function (Schulman and Harper, 2009), *Giardia*'s E1 showed the typical domains of this family (Fig. 1). It was found that the *e1* gene was expressed in proliferating trophozoites and its expression was differently regulated during encystations (Fig. 3). E1 mRNA was constitutively transcribed in growing cells but, when differentiation to cyst was induced, the expression increased, reaching its highest level 12 h after induction and then progressively decreasing its expression until the cyst was formed (Fig. 3A). Such behavior was also observed at the protein level (Fig. 3D) and represent the first evidence that E1 and ubiquitination might have an important role during *G. intestinalis* encystation.

E1 protein was studied using specific anti-E1 antibodies in wild type cells and in transfected cells overexpressing the enzyme. The results revealed several unusual characteristics, which are unique to this ubiquitin-activating enzyme. The E1 was translated as a full-length protein and then quickly proteolytically processed to generate two major peptides: E1-68 (N-terminal) and the E1-47 (C-terminal) (the names according with their approximate molecular weight) (Figs. 2C and 3C). These proteins were observed in immunoblots from trophozoites, cyst, all encystation stages and in cells transformed with the tagged protein.

In transformed trophozoites, that express a recombinant E1, the proteolytic event occurs as in normal cells since the full length protein was not detected, but an enhanced E1 activity was observed (reflected as a much higher Ub-conjugates production). The ubiquitination *in vitro* assay showed that E1 functionality was not affected by the proteolytic process of the enzyme. This processing of *Giardia* E1 in two polypeptides E1-68 and E1-47 strongly resembles the heterodimeric E1 proteins in the ubiquitination-like SUMO and NEED8 pathways. Members of the E1 family as SUMO- and NEED8-activating enzymes (E1s) are heterodimers as shown in Fig. 1 (Schulman and Harper, 2009) and have to be associated to generate the active E1. SAE1 (for SUMO) and NAE1 (for NEED8) are homologous to the N-terminal half of the E1 for Ub described here, and on the other hand UBA2 (for SUMO) and UBA3 (for NEED8) are homologous to the C-terminal half. Both subunits (MoeB or ThiF repeats) are important in generating the adenylation domain responsible for the Ub-like protein (Ubl) recognition (Lee and Schindelin, 2008; Schulman and Harper, 2009). By analogy to these heterodimeric E1 enzymes, and based in our results that show the absence of the complete protein both in normal and in transformed cells overexpressing E1, we suggest the possibility that *Giardia*'s E1 peptides: E1-68 and E1-47, associate to generate the active enzyme.

Immunofluorescence assays were performed to localize the enzyme during growth and differentiation to further characterize *Giardia* E1. As stated before, the assays showed that the antibodies generated recognized specific E1 forms (E1-114, E1-90 and E1-67 with anti-gNTE1; and E1-47 with anti-gE1CT); the patterns were very similar when using antibodies against the C or the N terminal fragments. The protein was localized at the cytoplasm in several small spots and not in a diffuse form (Fig. 4). The punctuate pattern may indicate E1 vesicular or membrane association. This specific pattern may also reflect regulation by ubiquitination in specifics compartments within the cell. When *Giardia* was induced to encyst, the protein presented a more diffuse pattern in the cytoplasm (Fig. 4). Encystation involves important molecular and cellular processes and regulation of the synthesis, sorting and transport of cyst wall components (Carranza and Lujan, 2010). The change in the localization pattern in encysting cells may be caused by association of ubiquitination to events involved in the process. Besides, we observed co-localization between some E1 signals and encystation-specific vesicles (ESVs) in encysting cells (Fig. 4), the ESVs are secretory granules which transport CWPs (cyst wall proteins) to the cell surface for release and assembly into the cyst wall (Carranza and Lujan, 2010). The protein was localized also as a diffuse pattern in the cyst cell body but, interestingly, also in the wall or very close to it. Such localization might be important because the only known antibodies to label the wall are those against CWPs. Other proteins had been localized in the cyst wall: A high cysteine non-variant cyst protein (HCNCp) and a group of (EGF)-like cyst proteins (EGFCPs), in these cases the authors used an epitope-tag approach for detecting those molecules in the cyst wall (Davids et al., 2006; Chiu et al., 2010). Localization in the cyst wall of E1 may show a relationship between ubiquitination and the dynamic formation and disruption of the cyst matrix, which is a poorly understood process.

It was thus found that E1 plays an important role in *G. intestinalis* biology. Inhibiting E1 expression by RNA antisense in trophozoites was lethal while E1 overexpression induced a five-fold increase in cyst formation during encystation. The changes in E1 location during this process and its localization in the cyst wall might show a role for E1 and ubiquitination in related cellular process. All the results presented here strongly indicated that E1, and ubiquitination itself, is essential for *Giardia* development and differentiation. The specific proteolytic process observed in E1 might be a clue in the evolution of this family of enzymes.

Acknowledgements

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