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Developmentally regulated expression of a *Giardia lamblia* cyst wall protein gene

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Summary

The protozoan *Giardia lamblia* is an obligate parasite of the mammalian small intestine. We studied the expression of a gene that encodes a protein component of the cyst wall, a complex structure assembled during the differentiation of trophozoites to cysts and which is critical to survival of the parasite outside its mammalian host. Transcripts from the cyst wall protein gene increase more than 100-fold during encystation, reaching a maximum between 5 and 24 hours after induction. Cyst wall protein expression also increases dramatically during encystation, and, prior to its incorporation into the nascent cyst wall, the protein is contained within the encystation-specific vesicles of encysting trophozoites. The sequence of the cloned gene predicts an acidic, leucine-rich polypeptide of M_r 26 000 that contains 5.3 tandemly arranged copies of a degenerate 24-amino-acid repeat. A hydrophobic amino-terminal peptide probably serves as the initial signal that targets this protein to a secretory pathway involving vesicular localization during encystation and, ultimately, secretion to form the cyst wall.

Introduction

Giardia lamblia, an enteric pathogen of humans and other mammals, is responsible for endemic and epidemic diarrhoea worldwide (reviewed by Adam, 1991). In addition to its medical significance, this binucleate amitochondrial flagellate represents one of the earliest branches in the eukaryotic line of descent (Sogin *et al.*, 1989; Hashimoto *et al.*, 1994) and, consequently, is of considerable biological interest. During its life cycle, *Giardia* assumes two forms. Actively dividing trophozoites colonize the upper small intestine and are associated with disease. In the course of infection, some trophozoites differentiate into cysts, which are excreted with the faeces and are responsible for transmission of the parasite among its mammalian hosts, largely via contaminated drinking water (reviewed by Craun, 1990). Cysts are distinguished by an outer wall, which is associated with resistance of these highly infectious forms to disinfection and hypotonic lysis (Sheffield, 1979). The cyst wall is composed of a fine fibrous network (Sheffield and Bjorvatn, 1977; Erlandsen *et al.*, 1989) and is antigenically and ultrastructurally distinct from the variant-specific surface proteins (VSPs; Mowatt *et al.*, 1991), which completely cover the trophozoite surface and may change during trophozoite growth (Gillin *et al.*, 1990; Nash *et al.*, 1988; Pimenta *et al.*, 1991).

Trophozoites differentiate to cysts by the process of encystation, which can be reproduced efficiently *in vitro*. Although bile or bile salts and a shift in pH (from 7.0 to 7.8) have been implicated in the induction of this developmental transition (Gillin *et al.*, 1987; 1989; Schupp *et al.*, 1988; Kane *et al.*, 1991), encystation also has been achieved *in vitro* under conditions of bile starvation (Sterling *et al.*, 1988). During differentiation, the encystation-specific vesicles (ESVs) appear and transport cyst wall components to the plasma membrane of encysting trophozoites (Reiner *et al.*, 1989; 1990; 1993). The formation of ESVs is regulated by exposure of trophozoites to encystation conditions that presumably mimic stimuli encountered in the small intestine (Faubert *et al.*, 1991). Although these and other reports (Ward *et al.*, 1990; Campbell and Faubert, 1994) clearly establish the appearance of cyst-specific antigens during encystation, the structure and biosynthesis of these antigens remain poorly understood. The identification and characterization of genes that encode cyst wall components would significantly advance our understanding of this complex and important structure; unfortunately, no genes encoding these or any molecules regulated during *Giardia* development have been reported.

We used a monoclonal antibody (mAb) that binds an antigen of the *Giardia* cyst wall (Stibbs, 1989) to study the expression of a cyst wall protein and to clone the corresponding gene. This antigen is diagnostically important,

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and mAb 5-3C can be used in conjunction with a polyclonal antiserum to diagnose infection using an antigen-capture enzyme-linked immunosorbent assay (Stibbs, 1989). Steady-state transcripts derived from the cloned gene, which we have named *CWP1*, are detectable in normal trophozoites at a low level but dramatically increase in abundance during differentiation to cyst forms. Synthesis of the protein, CWP, is induced in trophozoites during encystation *in vitro*, and it is concentrated in the ESVs of encysting trophozoites prior to its secretion to form the cyst wall. The deduced structure of CWP, in addition to experimental evidence reported here, provides important new insights into the structure of the cyst wall and its assembly.

Results

CWP is concentrated in encystation-specific vesicles in encysting Giardia *trophozoites*

Monoclonal antibody 5-3C exhibited a pattern of punctate cytoplasmic fluorescence in trophozoites recovered from the small intestine of infected gerbils (Stibbs, 1989), suggesting that CWP is concentrated in ESVs within trophozoites before it is incorporated into the cyst wall. We examined cells encysted *in vitro* by immunoelectron microscopy using mAb 5-3C and observed that CWP is concentrated in the osmiophilic and irregularly shaped ESVs (Fig. 1A) prior to its incorporation into the cyst wall (Fig. 1B). No labelling was observed if mAb 5-3C treatment was omitted or if purified non-immune mouse IgG1 was used as the primary antibody (data not shown). To examine the kinetics of appearance of CWP-containing vesicles during encystation *in vitro*, we performed immunofluorescence assays on permeabilized trophozoites at different times during encystation. Vesicles were not observed in cells cultured in growth medium (non-encysting trophozoites, Fig. 1C), but were found at a very low frequency in cells cultured in pre-encystation medium, i.e. growth medium lacking bile (data not shown). In contrast, the number of cells containing vesicles as well as the number of vesicles per cell increased with time in encystation medium (Fig. 1, D and E). Oval cyst forms were first detected after 6 h of encystation and accumulated over 48 h (Fig. 1F). Water-resistant cysts were first detected at 24 h. We observed no labelling of any sample if mAb 5-3C treatment was omitted.

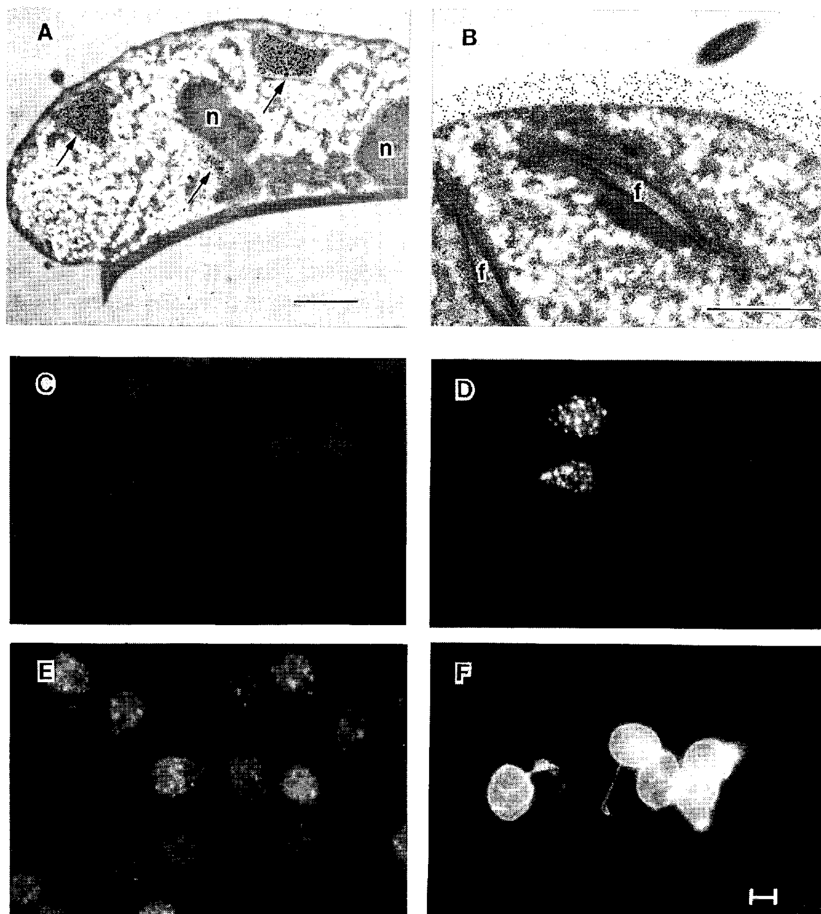

Fig. 1. Prior to its incorporation into the cyst wall, CWP is concentrated in encystation-specific vesicles in encysting *G. lamblia* trophozoites.
A and B. Immunoelectron microscopic analysis of encysting trophozoites and cysts using mAb 5-3C. The bars represent 1 μM. A. Portion of an encysting trophozoite. Arrows point to ESVs. The abundant glycogen in these cells is extracted by the immunostaining procedure. n, nucleus.
B. Portion of a cyst, showing label throughout the cyst wall. f, flagellar axoneme.
C to F. Indirect immunofluorescence analysis of non-encysting and encysting trophozoites using mAb 5-3C. The bar represents 5 μM.
C. Trophozoites cultured in growth medium (non-encysting trophozoites).
D. Encysting trophozoites (3 h after transfer to encystation medium).
E. Encysting trophozoites (7 h after transfer to encystation medium).
F. Cysts (24 h after transfer to encystation medium).

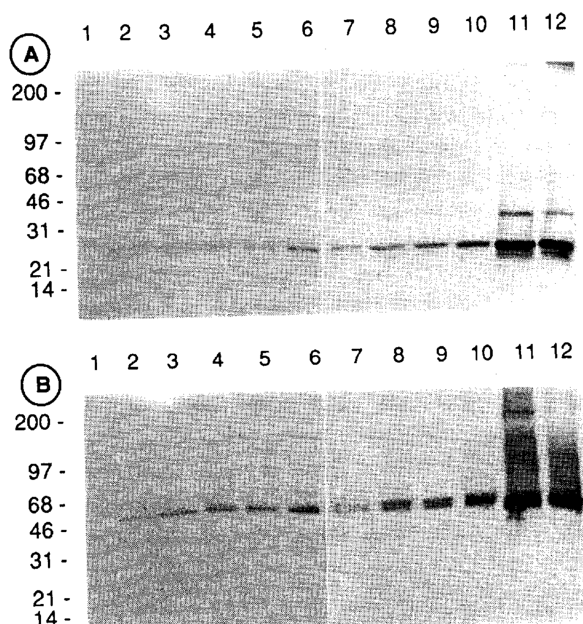

Fig. 2. CWP expression is upregulated during encystation *in vitro*. Immunoblot analysis of reduced (A) or unreduced (B) total trophozoite proteins (15 μg per lane) with mAb 5-3C. Lanes: 1, trophozoites cultured in growth medium; 2, trophozoites cultured in pre-encystation medium; lanes 3–12: trophozoites cultured in encystation medium for 60, 90, 120, 150, 180, 210 min, and 4, 5, 24 and 48 h, respectively.

CWP accumulates in a disulphide-linked form

We examined the expression of CWP during encystation by SDS–PAGE immunoblot analysis of reduced and unreduced total trophozoite proteins. When samples were reduced prior to electrophoresis, mAb 5-3C showed no reactivity with non-encysting trophozoites (Fig. 2A, lane 1). In contrast, in bile-starved organisms (trophozoites cultured for 72 h in pre-encystation medium) the antibody detected an approximately 26 kDa species which increased during exposure to encystation conditions (Fig. 2A, lanes 2–12). The kinetics of accumulation of the antigen were identical when the samples were not reduced prior to electrophoresis, but the protein migrated as an approximately 50 kDa doublet with significant upward smearing (retarded mobility) of antigen at later time points (Fig. 2B). The increased mobility and nearly complete elimination of smearing on reduction indicate the disulphide linkage of CWP to itself or to other protein(s). In addition to the 26 kDa species detected under reducing conditions, the mAb detected less-abundant species of approximately 44 and 24 kDa in the 24 h and 48 h samples (Fig. 2A, lanes 11 and 12). At 48 h, cyst forms and water-resistant cysts constitute approximately 80% of the cells (data not shown).

Steady-state levels of CWP mRNA increase more than 100-fold during encystation

A 595 bp cDNA fragment encoding a portion of CWP was cloned from an expression library using mAb 5-3C. Immunoblotting of crude and purified preparations of the recombinant protein verified its reactivity with mAb 5-3C and confirmed the failure of this antibody to bind bacterial proteins (data not shown). When used as a probe in RNA hybridization analysis, an antisense oligonucleotide derived from the cDNA sequence detected a transcript of about 900 nucleotides (nt) that increased in abundance during encystation (Fig. 3, CWP arrow). Long autoradiographic exposures (data not shown) indicated that the CWP transcript is present at a constant low level in non-encysting and bile-starved trophozoites (Fig. 3, lanes 1 and 2) and in trophozoites cultured for 60 min in encystation medium (Fig. 3, lane 3). However, the steady-state level of this RNA increased between 60 min and 7 h and decreased between this point and 48 h. In contrast, glutamate dehydrogenase (GDH) mRNA was detected in all

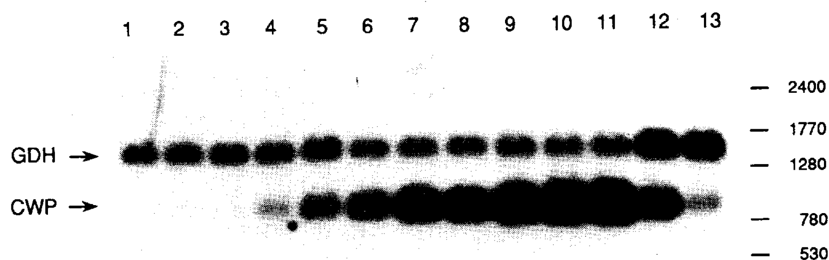

Fig. 3. CWP mRNA increases during encystation *in vitro*. Hybridization analysis of total trophozoite RNA (10 μg per lane) fractionated by 1.4% agarose/0.22 M formaldehyde gel electrophoresis. Lanes: 1, trophozoites cultured in growth medium; 2, trophozoites cultured in pre-encystation medium; 3–13: trophozoites cultured in encystation medium for 60, 90, 120, 150, 180, 210 min, and 4, 5, 7, 24 and 48 h, respectively. The filter was first hybridized to an antisense CWP oligonucleotide probe, subjected to autoradiography, then rehybridized with an antisense GDH oligonucleotide probe. Final post-hybridization washing conditions were 50°C, 2× SSC, 0.1% SDS. The positions of the GDH and CWP transcripts are indicated on the left; RNA ladder marker sizes in nucleotides are shown on the right.

samples (Fig. 3, GDH arrow). We quantified the CWP and GDH hybridization signals by phosphorimage analysis. GDH mRNA signals in lanes 1 to 11 varied from the calculated mean value by less than one standard deviation (3621 ± 588 arbitrary units), but increased approximately twofold by 24 h and 48 h (mean ± standard deviation for these time points was 7452 ± 223 arbitrary units). The ratio of CWP to GDH increased from 0.1 in non-encysting trophozoites to 10.5 after 7 h of encystation. Compared to trophozoites cultured in growth medium (lane 1), cells cultured in encystation medium for 7 h (lane 11) exhibited a nearly 140-fold increase in the level of CWP RNA.

CWP is a 26 kDa leucine-rich protein that contains a degenerate 24-amino-acid repeat

The sequence of the cloned cDNA and its size relative to the detected transcript suggested that it represented only a portion of the CWP gene. Genomic Southern hybridization using the cDNA probe indicated that the gene, which we called *CWP1*, was present as a single copy in the *Giardia* genome (data not shown); therefore, the complete gene was obtained from a genomic library. DNA sequencing of the cloned genomic gene copy revealed an open reading frame of 723 bp encompassing and identical to the portion contained in the cDNA clone (Fig. 4A). Assuming translation starts at nucleotide 1, the deduced amino acid sequence describes a leucine-rich (15 mole%) protein with a pI of 3.5. The deduced protein has a M_r of 26 000, in excellent agreement with the 26 kDa band identified in immunoblots (Fig. 2A). Hydropathy analysis indicated amino-terminal and tandem internal hydrophobic peaks (data not shown). Cleavage of a 14-residue amino-terminal signal peptide was predicted by

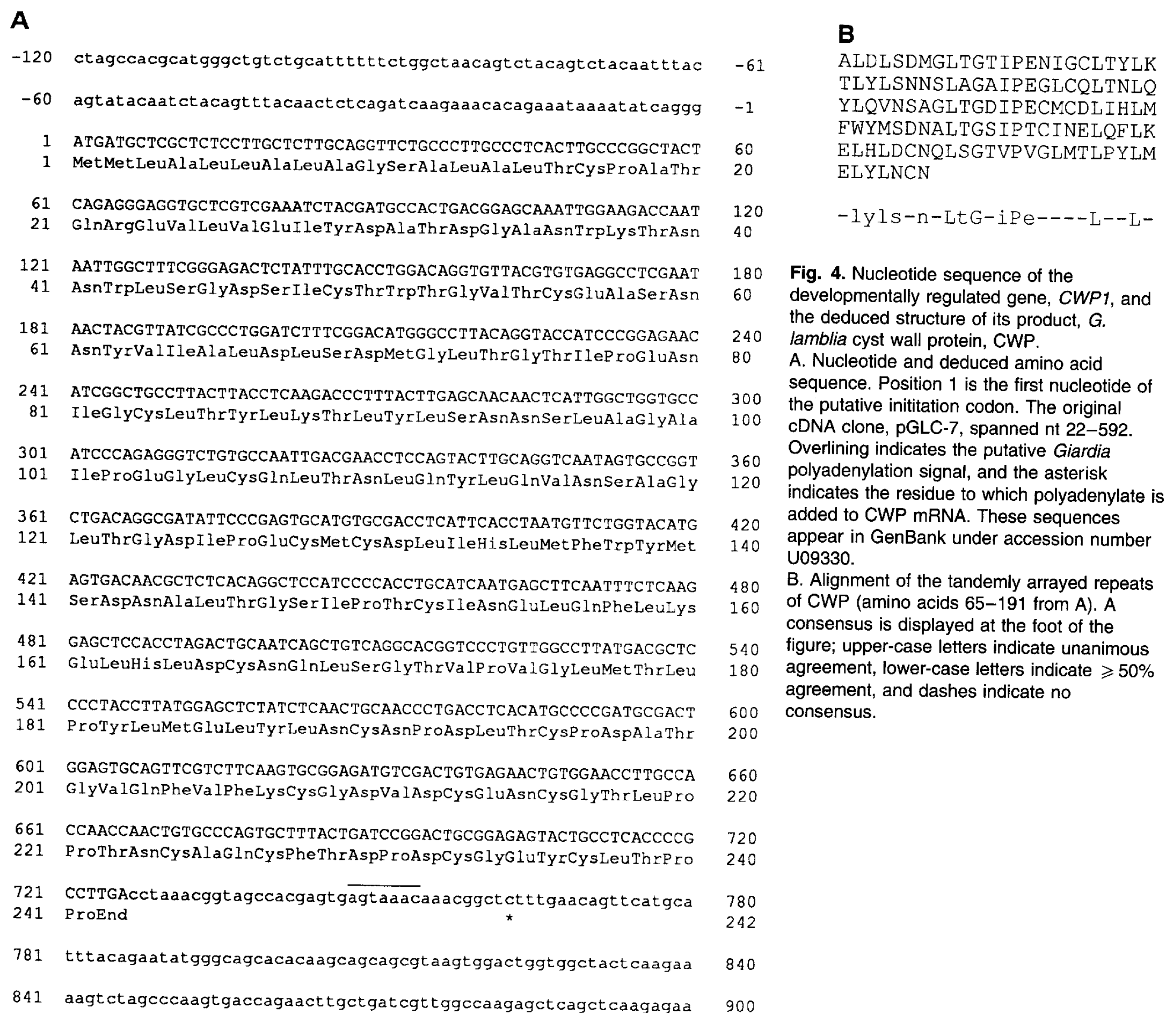

Fig. 4. Nucleotide sequence of the developmentally regulated gene, *CWP1*, and the deduced structure of its product, *G. lamblia* cyst wall protein, CWP.
A. Nucleotide and deduced amino acid sequence. Position 1 is the first nucleotide of the putative inititation codon. The original cDNA clone, pGLC-7, spanned nt 22–592. Overlining indicates the putative *Giardia* polyadenylation signal, and the asterisk indicates the residue to which polyadenylate is added to CWP mRNA. These sequences appear in GenBank under accession number U09330.
B. Alignment of the tandemly arrayed repeats of CWP (amino acids 65–191 from A). A consensus is displayed at the foot of the figure; upper-case letters indicate unanimous agreement, lower-case letters indicate ⩾ 50% agreement, and dashes indicate no consensus.

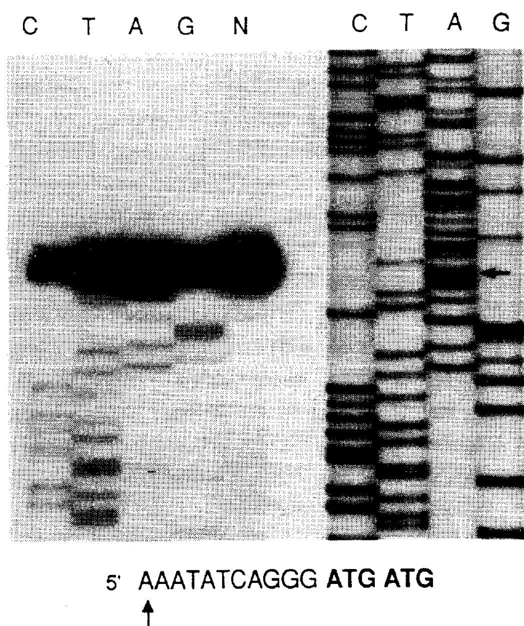

Fig. 5. Primer extension sequence analysis of the *CWP1* transcript. Chain termination sequencing reactions were performed as described in the *Experimental procedures* using RNA from trophozoites encysted for 7 h (same sample as lane 11 in Fig. 3). The lanes are labelled to allow reading of the sense strand (5′ to 3′ from top to bottom). Primer extensions on the RNA template are on the left, with lane N indicating a reaction performed in the absence of dideoxynucleotides; reactions using the same primer on the cloned gene are shown on the right. The arrows on the right and at the bottom of the Figure indicate the nucleotide that corresponds to the major run-off product as determined in a brief autoradiographic exposure. The sequence of the *CWP1* 5′ untranslated region and first two codons (bold type) is indicated at the foot of the Figure.

the algorithm of von Heijne (1986), and dot-matrix analysis confirmed the internal redundancy: CWP contains 5.3 tandemly arranged copies of a degenerate 24-residue repeat (Fig. 4B). Consistent with its potential to form disulphide bonds, CWP has 18 cysteine residues (7.5 mole%). In addition, a single consensus N-linked glycosylation site occurs within repeat 2 (amino acids 94 to 96).

Transcription initiates close to CWP1 *and yields polyadenylated mRNA*

Primer extension sequence analysis was used to establish the 5′ end of CWP mRNA and to verify the putative initiation codon identified by DNA sequencing. This analysis indicated that in encysting trophozoites *CWP1* transcription initiates proximal to the open reading frame to yield mRNA with a 5′ non-coding leader of approximately 11 nt (Fig. 5), the longest yet observed in *Giardia*, exceeding the 6 and 9 nt leaders observed for the ADP-ribosylation factor (Murtagh *et al.*, 1992) and triosephosphate isomerase (Mowatt *et al.*, 1994) mRNAs, respectively. Comparison of RNA from normal and encysting trophozoites revealed a quantitative difference but qualitative identity between the run-off products of primer extension (data not shown). 3′ RACE was employed to complete the characterization of the *CWP1* transcript and established that polyadenylation occurs nine nucleotides downstream from the heptanucleotide AGTAAAC (Fig. 4A), which conforms to the motif found invariably between the open reading frame and polyadenylation site of *Giardia* mRNAs (reviewed by Adam, 1991). Subtraction of the transcribed gene sequence (774 nt) from the mRNA size observed by hybridization (900 nt) suggests a polyadenylate tract of about 125 nt, which falls within the range observed in other eukaryotes (Darnell *et al.*, 1990).

Discussion

The differentiation of *Giardia* trophozoites to cysts is required for survival of the parasite outside the mammalian intestine and, therefore, is essential for the efficient dissemination of the infection among susceptible hosts. In this study we identified and characterized the gene *CWP1*, whose expression is regulated during this important developmental transition.

During encystation *in vitro*, steady-state levels of *CWP1* transcripts increased to a maximum nearly 140 times that observed in non-encysting trophozoites. As encystation *in vitro* is not synchronous, this increase was gradual and was first observed 90 min after transfer of the trophozoites from conditions of bile starvation (pre-encystation medium) to encystation medium. By 48 h, CWP mRNA levels dropped more than 10-fold relative to the maximum observed at 7 h. In contrast, over 7 h of encystation, GDH mRNA levels remained constant but had increased two-fold by 24 and 48 h. At 48 h, cyst forms, i.e. cells having completed cyst-wall assembly, constitute about 80% of cells, and of these approximately 80% are viable cysts. Since ESVs disappear upon assembly of the cyst wall (Reiner *et al.*, 1990; this report), the decrease in CWP mRNA level probably reflects the reduced number of cells actively synthesizing the cyst wall. The increase in mRNA encoding GDH, an important metabolic enzyme in *Giardia* (Paget *et al.*, 1993), may reflect the fact that two trophozoites ultimately emerge from each viable cyst; therefore, the GDH mRNA level per cell may remain constant.

Immunochemical studies indicate that some, if not all, cyst wall components are absent in non-encysting trophozoites (Reiner *et al.*, 1989; 1990; 1993; Ward *et al.*, 1990; Campbell and Faubert, 1994). Consistent with these observations, we failed to detect CWP in non-encysting trophozoites but found that its expression is strongly induced during encystation *in vitro*. In contrast to CWP mRNA, which first increased above non-encysting levels 90 min after transfer of trophozoites to encystation

medium, we observed production of CWP that began during pre-encystation and increased between 60 min and 24 h of growth in encystation medium. Taken together, these observations suggest that, prior to the dramatic increase in steady-state CWP mRNA levels observed later in encystation, CWP levels in non-encysting cells and very early in encystation may be controlled at the level of translation initiation, elongation and/or post-translationally by protein turnover. Presumably this control is relaxed when CWP mRNA levels increase. Clearly, the mechanisms responsible for effecting the increase in CWP mRNA levels as well as for controlling CWP synthesis remain to be established.

Immunoelectron microscopic analysis of encysting trophozoites confirmed an early suggestion that CWP was contained within vesicles prior to assembly of the cyst wall (Stibbs, 1989). The kinetics of appearance of these vesicles during encystation, their irregular shape and osmiophilic nature, as well as their contents (i.e. cyst wall components) are identical to the ESVs described by Reiner *et al.* (1989; 1990; 1993). Consistent with its vesicular localization during encystation and ultimate secretion from the cell, the primary structure of CWP strongly suggests its initial targeting to the secretory pathway by virtue of an amino-terminal signal peptide. Although we lack direct evidence for the cleavage of this peptide from CWP a precedent for the cleavage of amino-terminal peptides of similar amino acid composition and hydrophobicity exists in the cases of two cell-surface proteins of *Giardia* trophozoites, TSA 417 (Aley and Gillin, 1993) and VSP H7 (H. D. Luján *et al.*, submitted). We have not observed CWP associated with the plasma membrane and have failed to detect VSPs in the cyst wall or associated with the ESVs (data not shown). These results are consistent with the observations of Reiner *et al.* (1990), which indicate that *Giardia* is capable of intracellular protein sorting. If CWP contains a signal responsible for its targeting to the ESVs, the identification of such a signal may be possible by comparing the structures of CWP and other cyst wall proteins, once these genes have been identified and characterized.

Biochemical analyses suggest that the cyst wall consists of both carbohydrate and protein components (Ward *et al.*, 1985; Jarroll *et al.*, 1989; Manning *et al.*, 1992); however, the mechanism of cyst-wall assembly is completely unknown. High-resolution electron micrographs of the cyst wall reveal an elaborate fibrillar network composed of individual thin filaments (Erlandsen *et al.*, 1989). The filaments are of different lengths and thicknesses, the longest among them being characterized by multiple bifurcations and interconnections via short filaments. After SDS treatment, a fraction of the cyst wall remains insoluble (Jarroll *et al.*, 1989; Manning *et al.*, 1992), but purified cyst walls can be dissolved completely in 8 M urea with disulphide reducing agents (H. D. Luján, unpublished observations), indicating disulphide cross-linking of the wall constituents. Many components of the mammalian extracellular matrix (e.g. fibronectin and collagen Type VI) exhibit similar characteristics (McDonald, 1988). Fibronectin dimers are formed rapidly after synthesis by oxidation of cysteine residues, and our data suggest that this also occurs in the case of CWP. Later in encystation, during or following secretion of the ESV contents, multimerization and cyst-wall formation might be mediated by disulphide cross-linking of the CWP dimers. The composition of CWP (high leucine content and multiple cysteine residues), the presence of tandem amino acid repeats and the cellular localization of CWP support the idea that this protein plays an important role in the formation of the fibrillar component of the cyst wall.

Giardia encystation is a developmental process that results in the generation of quiescent and environmentally stable cell forms, and in this sense it resembles endospore formation in *Bacillus* and sporulation in *Saccharomyces* (reviewed by Losick and Shapiro, 1984). Sporulation in these and other microorganisms represents an adaptive response to a variety of factors that can include nutrient depletion, population density, and progression through the cell-division cycle. The exact stimuli responsible for *G. lamblia* encystation are not known, largely because of the lack of defined media for trophozoite growth and differentiation. The accumulation of transcripts from *CWP1* is an excellent marker for the induction of encystation and, in this regard, will be a useful tool not only for identifying factors that trigger or inhibit this important developmental transition but also for elucidating the molecular mechanisms that regulate the process.

Experimental procedures

Giardia *cultivation and encystation* in vitro

Trophozoites of the *G. lamblia* isolate WB (ATCC 30957), clone 1267 (WB/1267), were cultured in TYI-S-33 medium supplemented with 10% adult bovine serum and 0.5 mg ml^{-1} bovine bile (growth medium) as described previously (Nash *et al.*, 1988). Encystation of trophozoite monolayers was accomplished by the method described by Boucher and Gillin (1990). Briefly, 500 cells ml^{-1} were cultured for 72 h in medium without bovine bile (pre-encystation medium), the medium was discarded, and the tube containing a confluent monolayer of trophozoites was refilled with encystation medium (TYI-S-33, pH 7.8, containing 5 mM lactic acid and 250 $\mu g\ ml^{-1}$ of porcine bile). Viability of *Giardia* cysts was determined by staining with fluorescein diacetate and propidium iodine (Schupp and Erlandsen, 1987).

Immunofluorescence and immunoblot analysis

For immunofluorescence analysis, cells cultured in either

growth medium, pre-encystation medium or encystation medium were harvested by chilling the culture tubes and attaching cells to poly-L-lysine-coated glass slides at 37°C for 30 min in a CO_2 incubator. Adherent cells were rinsed twice with PBS, then fixed and permeabilized for 5 min with methanol:acetone (1:1) at −20°C. Slides were blocked for 30 min with 5% normal goat serum (Vector Laboratories) in PBS and then incubated for 1 h with mAb 5-3C (IgG1; Stibbs, 1989). After rinsing three times (15 min total), fluorescein-conjugated goat antisera (Organon–Teknika–Cappel) diluted in PBS/goat serum were added followed by three PBS washes. The specimens were mounted in Vectashield (Vector Laboratories) and viewed on a Zeiss Axiophot microscope.

For immunoblot analysis, protein concentration was determined according to Lowry (Lowry *et al.*, 1951). Total cell proteins were incubated for 6 min at 100°C in sample buffer with or without 5 mM 2-mercaptoethanol and then fractionated by SDS–PAGE in 4–20% gradient gels (Laemmli, 1970). Electrophoretic transfer of proteins to nitrocellulose was at 30 V for 10 h in 20 mM Tris, 150 mM glycine, 20% methanol (Towbin *et al.*, 1979). Filters were blocked with 3% defatted milk/0.1% Tween 20/PBS for 1 h and then incubated with mAb 5-3C (1:400 dilution) for 2 h. Following incubation of the filter with alkaline-phosphatase-conjugated goat-antimouse IgG (Southern Biotechnology Associates, Inc.) at 1:1000 dilution, CWP was visualized by development with Western Blue substrate (Promega).

Electron-microscopic immunolabelling

Encysting (12 h) *G. lamblia* were fixed by dilution of the encystation medium with 5 vols of 3% glutaraldehyde in 0.1 M sodium phosphate buffer, pH 6.8. Fixation was for 1.5 h at room temperature, followed by rinsing in phosphate buffer and enrobing in low-melting-point agar. Enrobed cells were post-fixed for 30 min at room temperature in 1% osmium tetroxide in 0.1 M sodium phosphate buffer, pH 6.8, and embedded in LRWhite (London Resin Co. Ltd). Thin sections on nickel grids were blocked for 30 min in 1% BSA in PBS (BSA/PBS) and then incubated for 2 h in a 1:500 dilution of mAb 5-3C in BSA/PBS followed by a thorough rinse in PBS. Sections were then incubated for 1 h in goat-antimouse IgG coupled to 10 nm gold particles (BioCell, Ted Pella) and rinsed in PBS and distilled water. The grids were stained with uranyl acetate and lead citrate and carbon-coated before examination in the electron microscope. Controls omitting the primary antibody or using purified non-immune mouse IgG1 (Southern Biotechnology Associates, Inc.) as the primary antibody showed no label.

Nucleic acid purification and hybridization analysis

Nucleic acids were extracted from freshly harvested trophozoites as described previously (Mowatt *et al.*, 1991) except that RNAzol™ B (Tel-Test, Inc.) was used to purify total RNA. Standard procedures were employed in the electrophoresis, blotting and hybridization of genomic DNA and total RNA (Sambrook *et al.*, 1989), but total RNA was fractionated electrophoretically through 1.4% agarose/0.2 M formaldehyde gels as described (Tsang *et al.*, 1993). ^{32}P-end-labelled antisense oligonucleotides oMM103 (5′-CAGGTGCAAATA-GAGTCTCC-3′; this report) and GDH9B (5′-TTCTCGTTCT-TGAGGTACAT-3′; nt 1080 to 1061 GenBank accession number M84604) (Yee and Dennis, 1992) were used in RNA hybridization studies. Hybridization signals were quantified by analysis on a PhosphorImager:SF using ImageQuant™ software (Molecular Dynamics).

Library construction, screening and subcloning

An expression library was constructed in λgt11 using total RNA extracted from a pool of two cell types: (i) cysts recovered from the faeces of infected gerbils; and (ii) trophozoites induced to encyst *in vitro*. First-strand cDNA synthesis was accomplished by extension of random hexamers. Approximately 300 000 unamplified plaques (78% recombinant) were screened with mAb 5-3C, and a single clone, λGLC-7, was identified. The 595 bp *Eco*RI insert of λGLC-7 was subcloned in pBluescript-II (Stratagene Cloning Systems) to yield plasmid pGLC-7. The gel-purified insert of pGLC-7 was labelled with digoxigenin-dUTP by random hexamer extension (Feinberg and Vogelstein, 1982) and used to screen a WB/1267 *Sau*3AI partial genomic library constructed in λFIXII (Mowatt *et al.*, 1991). A 6.5 kbp *Bam*HI fragment from genomic clone λGLC7-G2 was subcloned in pGEM-3Zf(+) (Promega Corporation) to yield plasmid pMM96.

DNA-amplification analysis

The site of mRNA polyadenylation was established by rapid amplification of cDNA ends (3′ RACE; Frohman *et al.*, 1988) using the gene-specific primer oMM95 (5′-TCTTGCAGGT-TCTGCC-3′). The 800 bp amplification product was cloned in pGEM-4 (Promega) to yield the plasmid pMM97.

DNA and RNA sequence determination and analysis

DNA sequences were determined from double-stranded recombinant plasmid templates using Sequenase Version.2.0 (United States Biochemical Corporation). Sequences were generated from pMM96 by 'primer walking' on both strands using oligonucleotides designed from the newly derived sequences and made on an Applied Biosystems DNA Synthesizer Model 392. The identity of each reported nucleotide was determined an average of twice on each strand. Primers oMM100 (5′-CC-TAGACTGCAATCAGC-3′) and oMM104 (5′-CCGCCTTGAC-CTAAACG-3′) were used to establish the polyadenylate addition site in pMM97.

DNA sequences were determined from 10 μg of total RNA by reverse transcriptase-mediated extension of primer oMM103 (described above) as noted previously (Mowatt *et al.*, 1991). DNA Strider™ 1.2 (Marck, 1988), AnalyzeSignalase 2.0.3 (Mantei, 1992) and programs in the GCG package (Devereux *et al.*, 1984) running on the NIH Convex System were used to analyse and format the data.

Acknowledgements

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