Membrane-associated dipeptidyl peptidase IV is involved in encystation-specific gene expression during *Giardia* differentiation

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Giardia is a flagellated protozoan that resides in the upper small intestine of its vertebrate host and is the most common cause of defined waterborne diarrhoea worldwide. *Giardia* trophozoites undergo significant biological changes to survive outside the host by differentiating into infective cysts. Encystation is thus essential for transmission of the parasite among susceptible hosts. In the present study, we report that bestatin, a competitive inhibitor of aminopeptidases, blocks cyst formation *in vitro* by abolishing the expression of encystation-specific genes, such as those coding for cyst wall proteins. Bestatin does not affect proliferating trophozoites, indicating that its effect is encystation-specific. Using biochemical and molecular biological approaches, we identified the enzyme inhibited by bestatin and cloned its corresponding gene. Sequence similarity indicated that this enzyme belongs to a family of dipeptidyl peptidases. Our results suggest that a specific proteolytic event caused by a constitutively expressed membrane-associated dipeptidyl peptidase IV is necessary for encystation of *Giardia*.

Key words: bestatin, cyst, parasite, protease.

INTRODUCTION

Giardia are flagellated, binucleated protozoa that parasitize the upper small intestine of a wide variety of vertebrate hosts. Human infections are caused by *G. lamblia* (synonym *G. intestinalis* and *G. duodenalis*), which is the most commonly reported intestinal parasite in the world [1,2]. In addition, *G. lamblia* represents one of the earliest lineages in the evolution of eukaryotic cells, as determined by comparative analysis of several genes [3,4]. Consistent with its evolutionary placement, *G. lamblia* has unusual biological characteristics, including the lack of mitochondria, the presence of two similar active nuclei and an atypical endomembranous system, as well as the absence of sexual stages of the life cycle [5,6].

Giardia has a simple life cycle, alternating between the trophozoite and the cyst forms [7,8]. Infections are initiated after ingestion of cysts from contaminated water or food supplies [9]. Excystation begins when the cysts go through the acidic environment of the host's stomach releasing the trophozoites, which proliferate and colonize the upper small intestine and are responsible for the clinical manifestations of the disease [1,2]. In the intestine, some trophozoites are induced to encyst, a process that consists of the regeneration of the extracellular cyst wall, allowing the parasite to survive outside the intestine and infect other susceptible hosts [5,6].

The differentiation process from trophozoites to cysts begins when trophozoites confront low concentrations of cholesterol in the inferior parts of the intestine [10]. Encystation is accompanied by the formation of typical secretory granules called encystation-specific vesicles, which transport cyst wall proteins (CWPs) [11,12] and carbohydrates [13,14] to the cell surface for release and assembly into the protective cyst wall [7,15,16]. Among the components of the cyst wall are two proteins, CWP1 and CWP2, that we have identified and characterized previously [11,12]. The expression of these two closely related proteins is induced co-ordinately during encystation and both are concentrated within encystation-specific secretory vesicles before their assembly into the fibrillar cyst wall [7].

The ability to induce *Giardia* encystation *in vitro* makes this organism an excellent model to learn about the processes involved in gene regulation and signal transduction [7,8]. For that reason, we are using this system to study the molecular mechanisms by which *Giardia* senses the stimulus for encystation and triggers differential expression of encystation-specific genes.

When the effects of several protease inhibitors on encystation were analysed [17], we observed that bestatin (Ubenimex), a competitive inhibitor of some aminopeptidases [18], not only blocks cyst formation, but also abolishes the expression of CWPs in a dose-dependent manner. Bestatin is a dipeptide that was first discovered in the culture supernatant of *Streptomyces olivoreticuli* [18] and a potent inhibitor of aminopeptidase B and leucine aminopeptidases [18,19]. In humans, bestatin has been shown to have anti-tumour and immunomodulatory activities most likely by inhibiting cellular peptidases [20–23]. In the presence of this inhibitor, *Giardia* appears insensitive to either cholesterol starvation or high bile concentration, which are both known to trigger encystation *in vitro* [24,25]. We consequently attempted the isolation and cloning of the molecular target of bestatin's effects. Using a bestatin-affinity matrix, we were able to purify an enzyme that was subsequently microsequenced. Molecular cloning allowed the characterization of the corresponding gene, which encoded a membrane-associated dipeptidyl peptidase (DPP) IV homologue. The effects of bestatin on *Giardia* encystation and the characterization of this enzyme strongly suggest that a specific proteolytic event is involved either in sensing the stimulus for encystation or during the signal transduction pathways implicated in transmission of the stimulus to the cell nuclei for differential gene expression during differentiation.

Abbreviations used: CWP, cyst wall protein; DPP, dipeptidyl peptidase; gDPP, *Giardia* DPP; mAb, monoclonal antibody; RT, reverse transcriptase.

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The nucleotide sequence of the *Giardia intestinalis* dipeptidyl peptidase IV has been deposited in the GenBank® Nucleotide Sequence Database under the accession number AF293412.

EXPERIMENTAL

Giardia lamblia cultivation and encystation *in vitro*

Trophozoites of the isolate WB, clone 1267 [26], were cultured in growth medium (TYI-S-33 medium supplemented with 10% adult bovine serum and 0.5 mg/ml bovine bile) as described previously [27]. Encystation of trophozoite monolayers was accomplished as described by Boucher and Gillin [28].

Assay of protease activity in cell cultures

The effects of bestatin on growth and encystation were analysed. Bestatin (Sigma) was dissolved in distilled water at 59 °C. To measure the effects of bestatin on encystation, trophozoites were grown in pre-encystation medium [28] for 2 days, the medium was then replaced with encystation medium [28] containing different concentrations of bestatin, and the cells were cultured at 37 °C for 24 h. Cysts and non-attached trophozoites were recovered from the medium and resuspended in tap water as described previously [28]. To quantify the inhibitor's effect on vegetative growth of the parasite, bestatin was added to the growth medium and cultures maintained at 37 °C for different time periods. Tubes containing attached trophozoites were chilled and the cells counted in a Coulter Z_1 Cell Counter.

Electrophoresis and immunoblotting

SDS/PAGE was performed under reducing conditions as described by Laemmli [29]. After electrophoresis, the gels were stained with Coomassie Brilliant Blue R-250. For Western blots, proteins were incubated in sample buffer with 2-mercaptoethanol and fractionated by SDS/PAGE on 12% (w/v) gels. Proteins were electrophoretically transferred on to nitrocellulose in 20 mM Tris/150 mM glycine/20% (v/v) methanol for 10 h at 30 V [30]. Filters were blocked with 3% (w/v) non-fat milk in Tris-buffered saline [25 mM Tris/HCl (pH 7.4), 140 mM NaCl and 3 mM KCl] containing 0.1% (v/v) Tween 20 and then incubated with the monoclonal antibody (mAb) 7D2 (specific for CWP2) [12] and mAb 5-3C (specific for CWP1; a gift from Dr H. H. Stibbs, Waterborne Inc., New Orleans, LA, U.S.A.) [11] at a 1:1000 dilution in Tris-buffered saline/0.1% (v/v) Tween/3% (w/v) non-fat milk. Following incubation, alkaline-phosphatase-conjugated goat anti-(mouse IgG) was added at a 1:2000 dilution. CWPs were visualized by using the Alkaline Phosphatase Color Development Reagent (Bio-Rad). The method of Lowry et al. [31] was used for protein quantification. Triton X-114 partitioning was performed as reported previously [17].

Immunofluorescence analysis

Cells cultured in grown medium, pre-encystation medium or encystation medium were harvested and processed as described previously [10]. Slides were then incubated with fluorescein-conjugated mAb 7D2. Specimens were mounted and viewed on a Leica IRME fluorescence microscope. The images were captured with a Leica DC250 camera and processed with Leica QFluoro Software (Leica Microsystems).

Enzyme purification and microsequencing

Non-encysting *Giardia* trophozoites (6×10^8 cells) were harvested and resuspended in 1 ml of PBS containing 0.1% Triton X-100, sonicated and centrifuged at 5000 ***g*** for 15 min. The supernatant was applied to a bestatin-affinity column prepared as reported previously [32]. Protein elution was carried out stepwise with 50 mM Hepes buffer (pH 6.8) containing 0.1 M, 0.25 M, 0.5 M and 1.0 M NaCl. Active fractions were pooled, dialysed and stored at −20 °C until use. Two purified proteins of 80 and 60 kDa obtained from bestatin-affinity chromatography were separated by SDS/PAGE and electrotransferred on to a PVDF membrane, which was briefly stained with Coomassie Brilliant Blue R-250. The stained bands were excised and subjected to Edman degradation on a PerkinElmer Biosystems Model 477A Gas-Phase Protein Sequencer by the Protein and Nucleic Acid Shared Facility, Medical College of Wisconsin, Milwaukee, WI, U.S.A. Only the 60 kDa protein could be sequenced.

Nucleic acid purification and analysis

Nucleic acids were purified and analysed by standard methods as described previously [10,11,33]. For reverse transcriptase (RT)-PCR, the first strand of cDNA synthesis was performed on total RNA extracted from encysting trophozoites by priming the template with oligo$(dT)_{20}$ and using avian myeloblastosis virus RT H^- (Promega). The amino acid sequencing of the N-terminus of the purified mature proteinase, together with knowledge of codon usage in *Giardia* [34], allowed the design of a moderately degenerate primer [5′-GA(C/T)TC(T/C)CA(T/C)-GG(C/G)CGGTA(T/C)GT(T/C)GC(G/C)TT(T/C)GTG-3′] encoding the amino acid sequence DSHGRYYAFV (where single-letter amino-acid notation has been used). For PCR, this 'guessmer' was used together with the oligo$(dT)_{20}$ primer. The PCR product was electrophoresed and gel purified using the QIAquick Gel Extraction Kit (Qiagen) and radiolabelled by using Prime-a-Gene System (Promega).

A *G. lamblia* cDNA library in λgt22a was screened as reported previously [10,11]. Duplicate lifts of the library were probed at 60 °C with the ^{32}P-labelled PCR product obtained from amplification by RT-PCR (see above) as reported previously [10,11]. λZAP II genomic DNA library screening was performed essentially as described previously [35]. DNA fragments were cloned into pBlueScript SKII+ and sequenced.

Fragments used as probes were purified from agarose gels and radiolabelled. For Slot blotting, 6 μg of total RNA extracted from growing trophozoites or trophozoites induced to encyst for different time periods were immobilized on to Hybond-N membranes (Amersham Biosciences) according to the manufacturer's instructions using Bio-Rad apparatus. In this case, membranes were hybridized with antisense probes specific for CWP1 and CWP2 [12,35]. For Southern-blot analysis, 10 μg of *G. lamblia* DNA was digested with several restriction enzymes and electrophoresed on 1% (w/v) agarose gels. DNA was transferred on to Hybond-N membranes using the TurboBlotter apparatus (Schleicher & Schuell). For Northern blotting, total RNA (10 μg per lane), isolated from trophozoites undergoing encystation for different time periods [10,11,35], was electrophoresed on 2.2 M formaldehyde/1% agarose gels and transferred on to Hybond-N membranes. Filters were hybridized as described previously [11] with the [α-^{32}P]dCTP random-primed-labelled full-length DPP gene. Final washings were done at 60 °C with 0.1 × SSC [where 1 × SSC is 0.15 M NaCl/15 mM sodium citrate (pH 7.0)] and 0.1% SDS. Membranes were then exposed to Kodak X-OMAT-AR films at −70 °C. Analysis of DNA sequences was performed with the computer program DNAStar (Lasergene). Signal sequence prediction was done using SignalIP [36]. SMART (Simple Modular Architecture Research Tools) [37] was used for searching for protein domains and patterns. Homology searches were performed with BLAST [38], and other structural predictions were produced with software available at ExPASy (http://www.expasy.ch/).

Expression and purification of recombinant *Gardinia* DPP (gDPP)

The cDNA fragment encoding gDPP, without the signal peptide sequence, was inserted into the *Eco*RI/*Bam*HI sites of pGEX-4T. The resulting construct encoding a glutathione S-transferase fusion protein was transformed into *Escherichia coli* strain M15 K12. Purification was done using the GST Gene Fusion System (Amersham Biosciences) as reported previously [17]. Purified recombinant gDPP was used to immunize mice [11], and pre-immune and immune sera were used for Western blotting and immunofluorescence as described above.

RESULTS

Bestatin inhibits *Giardia* differentiation

In the context of an extensive study regarding the involvement of proteolytic activities on *Giardia* encystation [17], we observed that the aminopeptidase inhibitor bestatin blocked cyst wall formation. For that reason, we tested the effects of different concentrations of bestatin on both encystation and vegetative growth. Bestatin inhibited *Giardia* differentiation in a dose-dependent manner when included in the encystation medium. No cysts were observed in culture supernatants when bestatin concentrations were higher than 50 μg/ml (Figure 1, upper panel). When these cells were washed twice and then re-cultivated in encystation medium in the absence of bestatin, cyst formation was almost the same as the control (results not shown), indicating that the inhibitor effects were reversible. In contrast, when bestatin was added to growth medium, neither cell viability nor proliferation were affected by the presence of the drug at concentrations that blocked encystation (> 50 μg/ml) (Figure 1, lower panel).

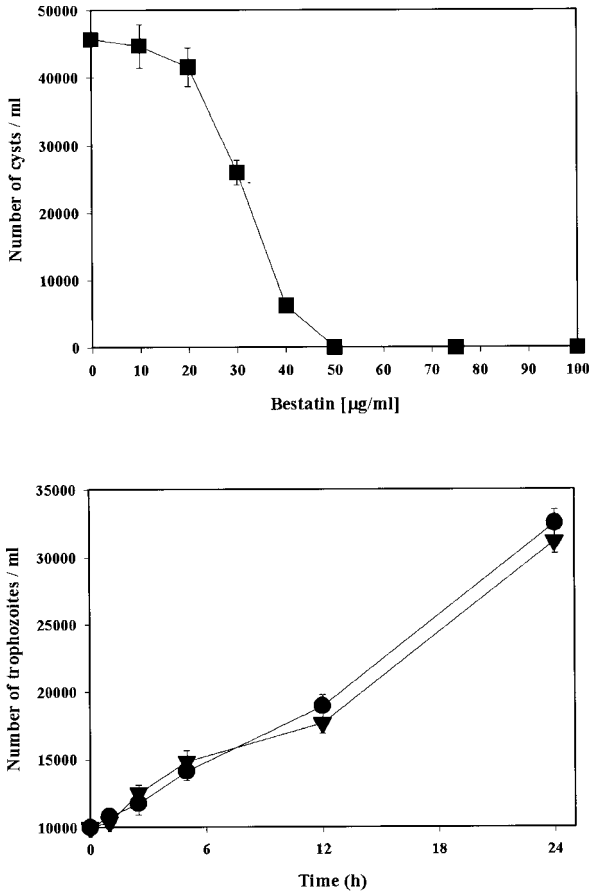

Figure 1 Effect of bestatin on *Giardia* is specific for encystation

Upper panel: *G. lamblia* trophozoites were cultured in encystation medium for 24 h in the absence or presence of different concentrations of bestatin, and the number of cysts produced was determined as described in the Experimental section. Bestatin concentrations over 50 μg/ml totally blocked cyst production. Lower panel: trophozoites were cultured in growth medium for different periods in the absence (▼) or presence (●) of bestatin (50 μg/ml). Attached cells were harvested and counted. Treatment with bestatin had no effect on cell proliferation at the concentration that blocked encystation. Results are the means ± S.D. of five independent experiments.

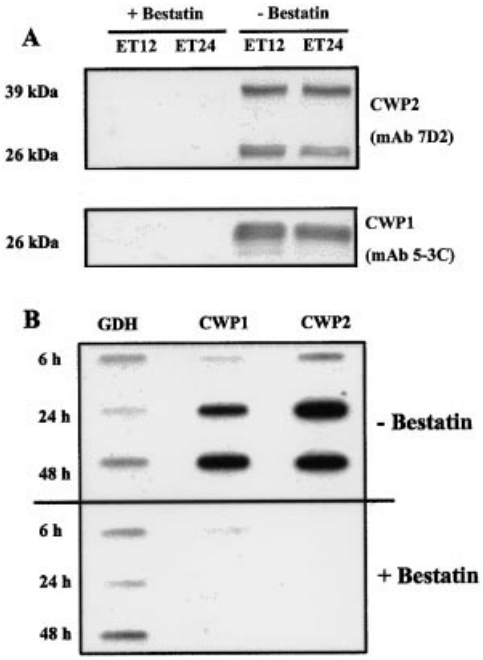

Figure 2 Bestatin abolishes the expression of CWP1 and CWP2 and their corresponding transcripts

(**A**) Western-blot analysis of *Giardia* trophozoites, induced to encyst for 24 h in the absence or presence of bestatin (50 μg/ml), using anti-CWP2 (upper panel) and anti-CWP1 (lower panel) mAbs. ET12 and ET24 indicate trophozoites induced to encyst for 12 and 24 h respectively. In the absence of bestatin, the 26 kDa band of CWP1 and the 26 and 39 kDa forms of CWP2 were detected. In the presence of bestatin, however, no expression of cyst wall proteins was observed. (**B**) Slot-blot analysis of total RNA extracted from trophozoites encysting for 6, 12 and 24 h in the absence or presence of bestatin. Probes specific for CWP1 and CWP2 and the housekeeping enzyme glutamate dehydrogenase (GDH) were used. In the absence of bestatin, there was a marked increase in the steady-state level of CWP transcripts compared with glutamate dehydrogenase. In contrast, in the presence of bestatin, there was no expression of mRNA encoding for CWP1 or CWP2.

Encystation of *Giardia* is thought to occur in three steps [7]: (1) the detection of the stimulus and activation of encystation-specific gene expression; (2) biogenesis of secretory organelles for transport of cyst wall materials, such as the Golgi complex and encystation-specific secretory granules; and (3) the release of the contents of the secretory granule and assembly of the extracellular cyst wall. To test at what stage bestatin blocked encystation, we first analysed the expression of two CWPs, CWP1 [11] and CWP2 [12], by Western blotting and indirect immunofluorescence by using specific mAbs. By Western blotting of encysting trophozoites, mAb 5-3C (specific for CWP1) detected a 26 kDa band and mAb 7D2 (specific for CWP2) recognized two polypeptides of 26 and 39 kDa respectively. By immunofluorescence, both antibodies labelled encystation-specific secretory granules that transport CWPs to the cell surface [11,12]. Figures 2(A) and 3

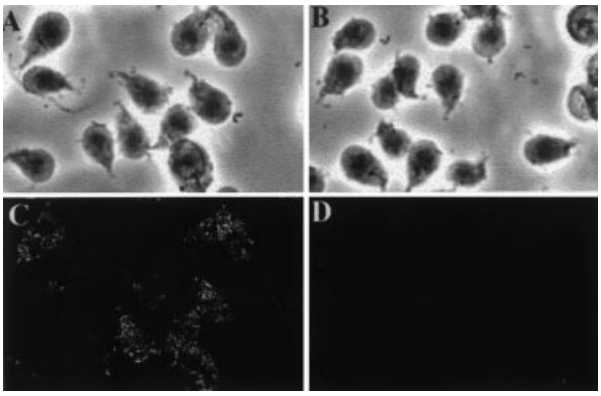

Figure 3 Bestatin abolishes the expression of CWP2

Indirect immunofluorescence assay showing that CWP2 was not expressed when bestatin was added to the encystation medium. mAb 7D2 against CWP2 was used to detect the presence of this protein in trophozoites induced to encyst in the absence (**A** and **C**) or the presence of 50 μg/ml bestatin (**B** and **D**). Cells were observed under phase contrast (**A** and **B**) and indirect imunofluorescence (**C** and **D**) microscopy. Scale, 1 cm = 40 μm.

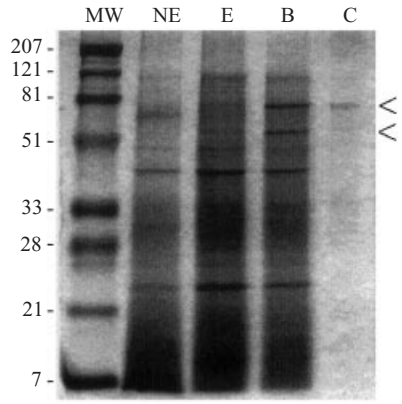

Figure 4 Expression of two proteins is increased when bestatin is added to culture medium

SDS/PAGE of *Giardia* proteins was performed as described in the Experimental section. NE, non-encysting trophozoites; E, encysting trophozoites; B, trophozoites cultured in the presence of bestatin (50 μg/ml). Arrowheads indicate the bands that increased in the presence of the inhibitor and proteins purified using bestatin–Sepharose chromatography (C). Molecular-mass markers (MW; in kDa) are indicated on the left.

show that no expression of CWPs occurred in cells maintained in encystation medium in the presence of 50 μg/ml bestatin. Controls in the absence of bestatin showed marked induction of the expression of these two CWPs. To analyse the level of transcription of the CWP genes (Figure 2B), we observed that, in the presence of bestatin, no expression of CWP mRNAs took place, indicating that this inhibitor was acting before specific gene activation was initiated, most likely by either making *Giardia* insensitive to the stimulus for encystation or blocking the transmission of the sensed signal to the nuclei of the parasite.

Interestingly, comparative analysis of total trophozoite proteins from vegetative and encysting trophozoites cultured in the presence of 50 μg/ml bestatin showed a marked increase in the expression of two proteins of 80 and 60 kDa when compared with controls without bestatin (Figure 4). This observation suggests that *Giardia* responds to the presence of this inhibitor by increasing the expression of the enzyme.

Enzyme purification, partial characterization and amino acid sequencing

The *Giardia* aminopeptidase was purified from both encysting and non-encysting trophozoites using a bestatin-affinity matrix. Two polypeptides of approx. 80 and 60 kDa were eluted from the column, in agreement with the molecular size of the two proteins whose levels were increased in the presence of the inhibitor (Figure 4, compare lanes NE and B). Preliminary characterization of the purified 60 kDa protein using fluorogenic amino-4-methyl courmarin ('AMC') peptide substrates indicated that it had bestatin-sensitive DPP activity (results not shown). Subsequently, both proteins were transferred on to a membrane and submitted to Edman degradation and amino acid sequencing of their N-terminal domains. No results were obtained from the higher-molecular-mass band; however, the 60 kDa protein presented the N-terminal sequence DSNGRYVAFV (where single-letter amino-acid notation has been used).

Molecular cloning of the purified *Giardia* peptidase

The amino acid sequence obtained from the lower-molecular-mass purified enzyme allowed us to design a moderately degenerate oligonucleotide primer that was then used in an RT-PCR reaction with total RNA obtained from encysting trophozoites. The PCR product was then used to screen both cDNA and genomic DNA libraries. Sequencing of positive plaques allowed the reconstruction of the full-length nucleotide sequence encoding a protein that contained an amino acid sequence identical with that obtained from the N-terminus of the purified 60 kDa protein. The nucleotide sequence is available in the GenBank® database under the accession number AF293412.

The open reading frame of this gene contained 2283 bp that code for 761 amino acids, accounting for an estimated size of 88 kDa with an isoelectric point of 4.78. The translated product started with a short signal sequence of 15 amino acids. This site is 256 residues upstream of the N-terminus determined by protein microsequencing of the 60 kDa polypeptide purified by bestatin-affinity chromatography. Since this methodology also allowed the purification of another protein of approx. 80 kDa, it is possible that both polypeptides correspond to the same molecule, which is proteolytically processed either physiologically or during its purification.

Transmembrane region prediction [37] indicated that the protein has a transmembrane domain between amino acids at positions 29 and 47 (Figure 5). Membrane topology is also supported by the fact that the same polypeptides were purified from the detergent phase of Triton X-114 partitioning of encysting trophozoites, and that a polyclonal antibody generated against recombinant gDPP labelled those peptides in Western-blot analysis (Figure 6A) and the plasma membrane of encysting cells by indirect immunofluorescence (Figure 6B).

A BLAST [38] homology search revealed that the enzyme shares sequence similarity with members of the DPP IV family of proteinases (E.C.3.4.14.5), and for this reason was called gDPP. Among these, gDPP possesses higher similarity with DPPs from other primitive eukaryotes, such as *Entamoeba histolytica* (NCBI Protein database accession number AAF20264), *Entamoeba dispar* (NCBI Protein database accession number AAF20265) and several fungi. Amino acid identity of gDPP, as shown by comparison of its putative translation product with its homo-

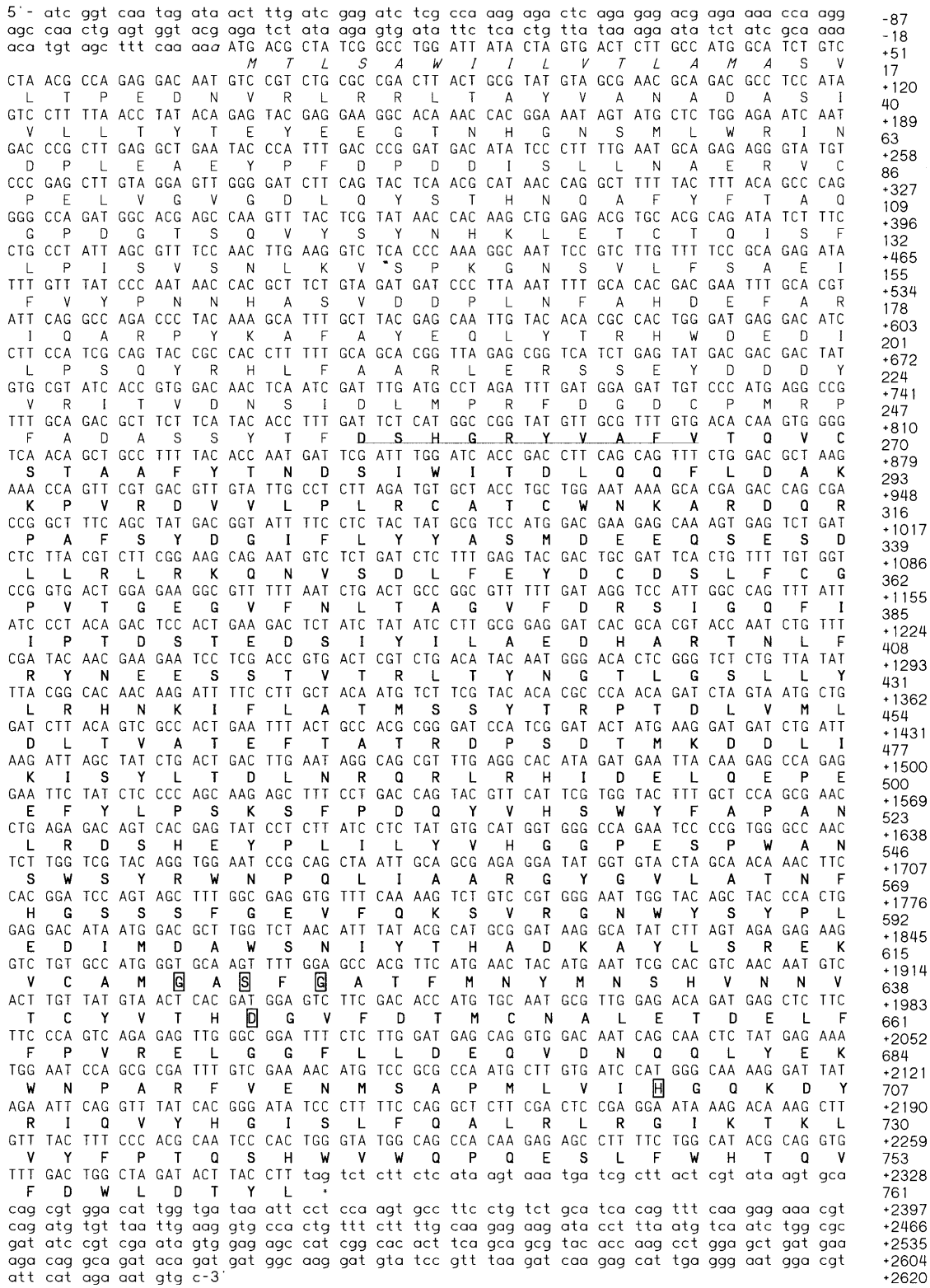

Figure 5 Domain structure of the gene encoding gDPP and its putative product

The sequence of the full-length gene encoding gDPP, with the open reading frame in capitals, is shown. The asterisk indicates the stop codon; amino acids at positions 1–15 of the translated gene, corresponding to the hydrophobic domain, are in italics; the residues of the 60 kDa bestatin-affinity purified protein are in bold; the amino acids at positions 257–266, corresponding to the sequenced N-terminal of the purified protein, are underlined; and the residues of the catalytic site (Gly^{620}, Ser^{622}, Gly^{624}, Asp^{645} and His^{702}) are boxed. The catalytic region is between amino acids at positions 390–761.

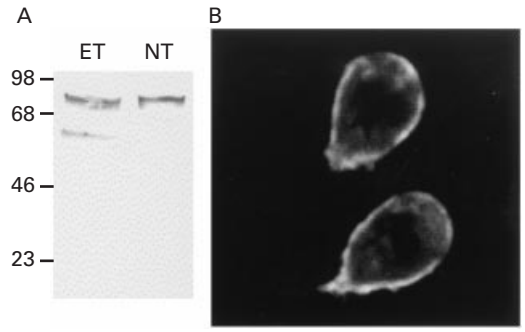

Figure 6 Immunolocalization of gDPP on the trophozoite plasma membrane

(**A**) Western-blot analysis, using a mouse polyclonal antibody generated against recombinant gDPP produced in *E. coli*, of the detergent phase of Triton X-114 extraction from encysting (ET) and non-encysting (NT) *Giardia* trophozoites. Molecular-mass markers (in kDa) are indicated on the left. DPP partitions into the detergent phase in both stages of the life cycle of the parasite. (**B**) Immunofluorescence of encysting trophozoites was performed using the same antibody as described in the Experimental section. No labelling was observed with the pre-immune serum. Scale, 1 cm = 20 μm.

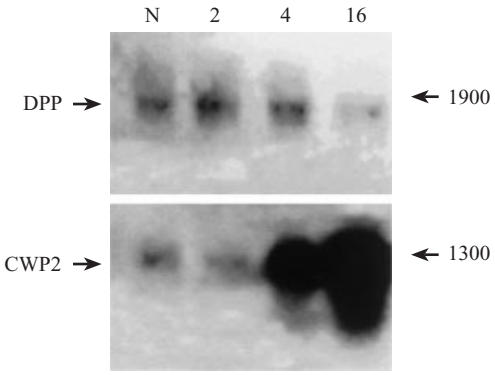

Figure 7 Expression of gDPP mRNA during *Giardia* encystation

Northern-blot analysis of trophozoites cultured in growth medium (N), or encystation medium for 2, 4 and 16 h. The same membrane was blotted with probes for CWP2 (lower panel; positive control for the encystation) and DPP mRNA (upper panel). Whereas the expression of CWP2 increases more than 200-fold during encystation, gDPP shows a slight decrease in the steady-state level of its transcripts.

logues from other eukaryotes, was in the 28–35 % range (results not shown).

Northern-blot analysis of gDPP mRNA expression (Figure 7) indicated that transcripts of this enzyme were present throughout the *Giardia* life cycle. Nevertheless, a slight decrease in the relative amount of gDPP mRNA was observed at the later times of encystation, suggesting again that this peptidase plays an important role during the earlier stages of the encystation process.

DISCUSSION

Giardia trophozoites undergo important biological changes to survive outside the intestine of their host by differentiating into environmentally resistant cysts. Encystation is thus essential for transmission of the parasite among susceptible hosts [1,2]. Since Gillin et al. [24] induced *Giardia* encystation *in vitro* for the first time, several molecular and cellular aspects of this process have been discovered [5,7,8]. We have previously clearly demonstrated that cyst formation *in vitro* is induced when trophozoites are starved of cholesterol [10], and proposed two non-exclusive models to explain this process. The first model suggests that cholesterol deprivation induces changes in the plasma membrane of trophozoites with subsequent activation of signal transduction pathways, which culminate in the expression of encystation-specific genes [7]. The second hypothesis is based on data about cholesterol regulation of transcription in mammalian cells [39], where repression of transcription by cholesterol is regulated at the DNA level by a 10 bp sterol-regulatory element, which is located in the promoter region of the genes encoding the low-density lipoprotein ('LDL') receptor and cholesterol-synthesizing enzymes [39,40].

Membrane fluidity of plasma membranes is influenced by the concentration of cholesterol in the environment. Modifications of the cholesterol content of membranes can control many membrane-related activities, such as membrane permeability, enzymic activities and receptor and sensor functions [41,42]. Since cholesterol depletion in *Giardia* culture medium affected changes in the fluidity of the trophozoite plasma membrane, we suggest that this modifies an enzymic activity that, in turn, might have important ramifications for the transduction of signals to the cell's nucleus.

The characterization and analysis of two CWP (*CWP1* and *CWP2*) genes that are developmentally regulated during encystation have been reported [11,12]. Interestingly, *CWP1* and *CWP2* genes are located in different chromosomes [7], indicating that their expression must be regulated at the level of transcription or mRNA stability, facts that also suggest that a specific signal must be directing this process.

In the present study, we have examined the effect of bestatin on *G. lamblia* differentiation and show that this dipeptide inhibitor prevents cyst formation most likely by inhibiting the activity of a membrane-associated DPP. Bestatin [18,19] was able to abolish expression of encystation-specific genes necessary for cell wall formation, but had no effect on proliferating trophozoites. These results strongly suggest that a parasite peptidase sensitive to bestatin is involved in encystation.

Aminopeptidases are hydrolytic enzymes that catalyse the removal of N-terminal residues from a polypeptide chain. Although the effects of bestatin as an anti-tumourogenic agent, macrophage activator and growth inhibitor for micro-organisms do not seem to result exclusively from the inhibition of aminopeptidase activities [22], its effects appear to be selective in *Giardia*. In the present study, the only two polypeptides that bind to bestatin are up-regulated in the presence of the drug, suggesting that the parasite responds to this competitive inhibitor by increasing the expression of the enzyme. This occurs in both growing and encysting trophozoites, which indicates that gDPP is present all through the *Giardia* life cycle.

We were also able to purify the enzyme by affinity to a bestatin column. Subsequently, this allowed the molecular cloning of the full-length gene encoding this peptidase. BLAST analysis indicated that the amino acid sequence encoded by the cloned gene shows highest similarity with *E. histolytica* and *E. dispar* DPPs belonging to the DPP IV group. It also has similarity with DPPs from *Schizosacharomyces pombe*, *Caulobacter crescentus*, *Xylella fastidiosa* and *Aspergillus fumigatus*. This similarity is located at the C-terminal end of the enzyme that contains the consensus site Gly^{620}-Xaa-Ser^{622}-Xaa-Gly^{624} and the putative catalytic triad of the DPP IV arranged in the same topological order (Ser^{622}, Asp^{645} and His^{702}). This is the first time that a DPP IV has been purified using bestatin-affinity chromatography, a system frequently used to isolate aminopeptidases. DPPs have been classified in six major groups (I–VI) on the basis of their preference for particular amino acids in P1 and P2 positions from

the susceptible peptide bonds, although there are some DPPs that do not fit this classification. DPP IV belongs to the S9 family of serine proteases that cleave dipeptides from the N-terminus of oligopeptides and proteins behind a penultimate proline or alanine residue. It is important to note that a peptidase from *A. fumigatus*, with homology to gDDP, has been classified as DPP V, due to its different catalytic properties [43].

Several proteinases have been isolated and characterized previously from *Giardia* [17,44–48]. They belong mostly to the cysteine protease family and some of them have been implicated in encystation [17] and excystation [48] of this parasite. However, no DPP IV has been described previously in *Giardia*. DPPs from lower eukaryotes have been suggested to play roles in food degradation and peptide metabolism, but none of these enzymes are related to cellular differentiation or signal transduction pathways. In humans, however, DPPs have fundamental physiological functions [21–23,43,49]. For instance, Xaa–Pro dipeptides from the N-termini of peptides may generate biologically active peptides or, in contrast, may inactivate peptides by truncation [49]. The DPP CD26 is a surface differentiation marker involved in the transduction of mitogenic signals in lymphocytes [23].

We still do not know the specific role of gDPP in trophozoite differentiation into cyst. However, we can hypothesize that this enzyme, located at the surface of trophozoites via a transmembrane domain in the N-terminal region of the protein, is in an inactive conformation during vegetative growth, since the effects of bestatin are only evident in encysting trophozoites. It is known that bestatin can bind to the surface of several mammalian cells, because several aminopeptidases are associated with the plasma membrane in various cell types [21]. When trophozoites confront a cholesterol-poor environment, they change the fluidity of their membranes and probably expose the active site of the enzyme, initiating a still unknown signal transduction pathway, which culminates in the induction of the expression of encystation-specific genes. Remarkably, although the function and structure of natural peptides that are cleaved by DPP IV is very diverse, most of them have in common the fact that they bind to, and signal through, prototypic G-protein-coupled heptahelical receptors [50–52]. Interestingly, bestatin possesses inhibitory activity over the growth of malaria micro-organisms [53], suggesting that the target of this drug might play important roles in the biology of other parasites as well.

Besides its medical importance, *Giardia* is considered an excellent system to study the evolution of fundamental cellular processes, since it belongs to the earliest branch of the eukaryotic lineage of descent [3,4]. As a consequence, encystation may represent an adaptive response developed by eukaryotes early in evolution to survive harmful conditions. Further studies of the role of gDPP described in the present study and the signal transduction mechanisms involved in differentiation might provide new insights about the evolution of adaptive processes in eukaryotic cells.

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