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The molecular characterization of the major polar tube protein gene from *Encephalitozoon hellem*, a microsporidian parasite of humans¹

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Abstract

The microsporidia are obligate intracellular protozoan parasites of increasing importance as human pathogens, which are characterized by a small resistant spore with a single polar filament that coils around the sporoplasm. When stimulated, the polar filament rapidly everts out of the spore to form a hollow polar tube through which the sporoplasm passes, thus serving as a unique mechanism of transmission. A genomic library of the human microsporidium *Encephalitozoon hellem* was screened using a polyclonal rabbit antibody (anti-PTP Eh₅₅) produced to the major HPLC purified polar tube protein (PTP) of *E. hellem*. This antibody localized to intrasporal polar filaments and extrasporal polar tubes of *E. hellem* by immunogold electron microscopy confirming the polar tube specificity of the antibody. A total of 14 anti-PTP Eh₅₅ reactive genomic clones were identified and purified. A PTP gene was identified consisting of 1362 bp coding for 453 amino acids. The N-terminus of the translated protein consists of a putative N-terminal signal sequence of 22 amino acids, which when cleaved results in a mature protein of 431 amino acids with a predicted molecular mass of 43 kDa. The protein has a high proline content (14.6%) and contains a central domain of six alternating tandem repeats of 20 amino acids. After ligation of the gene into a glutathione *S*-transferase (GST) expression vector, a fusion protein was produced that reacted by immunoblotting with the polar tube specific anti-PTP Eh₅₅. The gene was present as a single copy in the genome and there was no homology with other known genes. As the polar tube is a critical structure for the transmission of this organism to a new host cell, further study of PTPs may lead to the development of new therapeutic strategies and diagnostic tests.

¹Note: Nucleotide sequence data reported in this paper is available in the EMBL, GenBankTM and DDJB data bases under the accession number AF044915.

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Keywords

Microsporidia; *Encephalitozoon*; Polar filament; Polar tube; Polar tube protein; Polar tube protein gene

1. Introduction

Microsporidia are eukaryotic, obligate intracellular parasites defined by the production of a small spore containing a single polar filament that coils around the interior of the spore. When appropriately stimulated, the polar filament rapidly discharges from the spore forming a hollow polar tube that remains attached to the spore. Sporoplasm passes through this tube facilitating transmission of the parasite into a new host cell. Polar tubes resist dissociation in detergents and acids but dissociate in the presence of thiol reducing agents such as 2-mercaptoethanol and dithiothreitol (DTT) [1,2]. We previously isolated polar tube proteins (PTPs) from the spores of the fish microsporidian, *Glugea americanus*, after sequentially extracting glass bead disrupted spores with 1% SDS and 9 M urea, followed by solubilization of residual polar tubes in 2% DTT [3]. SDS-PAGE analysis demonstrated that the DTT solubilized material contained four proteins of 23, 27, 34 and 43 kDa [3]. A monoclonal antibody, mAb 3C8.23.1, reactive to the 43 kDa protein by immunoblotting, localized to the polar tubes of *G. americanus* by immunogold electron microscopy indicating it was polar tube in origin [3]. This 43 kDa protein was purified to homogeneity using reverse phase high performance liquid chromatography (HPLC), and it retained its immunoreactivity with the polar tube mAb [4]. Amino acid analysis revealed this protein to be rich in proline [4]. Polyclonal serum from mice, immunized with the purified 43 kDa PTP, reacted with a 43 kDa protein in *G. americanus* spore lysate by immunoblotting, and with extruded polar tubes and intrasporal polar filaments of *G. americanus* by immunogold electron microscopy [4].

In the present study, we applied our PTP isolation protocol to the purification of PTPs from the microsporidium *Encephalitozoon hellem*, an opportunistic pathogen in humans with AIDS, and used this purified protein to produce and characterize a polyclonal PTP antibody. The genomic size of *Encephalitozoon cuniculi* has been demonstrated to be 2.9 Mb [5], which is the smallest eukaryotic nuclear genome so far identified, and no introns have been found in microsporidian genes sequenced to date [6,7]. This suggests that expression screening of genomic libraries is feasible. The *E. hellem* PTP antibody was therefore utilized to screen an expression genomic library of *E. hellem* to identify the corresponding PTP gene.

2. Materials and methods

2.1. PTP purification and polyclonal antibody production

Rabbit kidney cells, RK13-CCL37 (American Type Culture Collection, Rockville, MD), infected with *E. hellem*, were maintained in continuous culture in minimum essential medium supplemented with 10% heat-inactivated fetal calf serum and 1% penicillin/streptomycin as previously published [8,9]. Spores (7×10^8 – 1×10^9) were glass bead-disrupted and sequentially extracted with 1% SDS and 9 M urea, and solubilized in 2% DTT as previously described [3,4,9]. The DTT-solubilized proteins were subjected to reductive alkylation using 4-vinylpyridine [4,10] followed by reverse phase HPLC on a Hewlett Packard Series II 1090 liquid chromatograph using a C8 column (Aquapore RP300, 220×2.1 mm, Applied Biosystems) as previously described [4]. PTPs were subjected to SDS-PAGE using 10% polyacrylamide followed by silver staining (2D-Silver Stain, Daiichi, Tokyo, Japan). Amino acid analysis was performed by Dr Alan J. Smith (Stanford University

Medical Center, Stanford, CA) using acid hydrolysis, a cationic column and post column derivatization with ninhydrin and colorimetric detection. N-terminal sequencing was performed by Edman degradation (Applied Biosystems 477A Protein Sequencer) on ≈ 100 pmol of the purified polar tube protein by the Laboratory for Molecular Analysis (Albert Einstein College of Medicine, Bronx, NY). Polyclonal antibody to polar tube protein (anti-PTP Eh₅₅) was produced by intradermal immunization of a New Zealand rabbit with 52 μ g of the purified PTP of *E. hellem* utilizing Hunter's TiterMax Adjuvant (CytRx, Norcross, GA).

2.2. Immunoblotting and immunogold electron microscopy

Proteins such as HPLC purified PTP of *E. hellem*, *G. americanus* or expressed recombinant PTP Eh₅₅ were resolved by SDS-PAGE (10% polyacrylamide) according to the procedure of Laemmli [11], electroblotted to nitrocellulose and incubated with a 1:2500–1:5000 dilution of anti-PTP Eh₅₅. Immune complexes were visualized using a 1:5000–1:10000 dilution of alkaline phosphatase conjugated secondary antibody and a chemoluminescent detection system using CSPD® (Western Light, Tropix, Bedford, MA). Immunogold electron microscopy was performed on intact and glass bead-disrupted spores, fixed and processed as previously published [3] using a 1:25 dilution of anti-PTP Eh₅₅.

2.3. Construction and screening of a genomic library

E. hellem spores were germinated by incubation with 0.3% H₂O₂ overnight at 25°C and genomic DNA was then phenol/chloroform extracted and ethanol precipitated. A total of 10 μ g of DNA from this preparation was partially digested with 2 U of *Sau*3AI at 37°C for 7 min and the DNA was size fractionated by agarose gel electrophoresis. Fragments ≥ 400 bp were then ligated into the *Bam*HI site of ZAP Express (Stratagene, La Jolla, CA). The resultant library had a titer of 2.6×10^6 plaques ml⁻¹, and 160000 plaques were screened with polyclonal anti-PTP Eh₅₅ using standard conditions (Protoplot, Promega, Madison, WI) [12]. Inserts of interest were excised out of antibody reactive, plaque-purified phage clones as kanamycin-resistant pBK-CMV phagemid vectors using the ExAssist interference-resistant helper phage and XL0LR bacterial strain of *E. coli* (ZAP Express, Stratagene). Plasmid DNA was subsequently purified using Wizard Plus (Promega). Gene copy was determined by restriction digestion of genomic DNA from *E. hellem* with *Eco*RI, *Pst*I, *Bam*HI, *Hind*III, *Sac*I, *Not*I, *Bgl*II and *Sma*I followed by Southern blotting using ³²P random primed (Boehringer Mannheim, Indianapolis, IN) PTP-Eh₅₅ gene (1.3 kb insert) as a probe [12].

Clones were sequenced on a ABI Prism Model 377 DNA sequencer (Perkin Elmer, Foster City, CA) using T7 and T3 oligmers flanking the insertion site as well as additional 18–20 bp oligomers selected using Oligo Primer Analysis Software (NBI, Plymouth, MN) based on the observed sequence data. Sequence data sets were assembled using the SeqMan program and the predicted protein analyzed using the Protean program of the LaserGene Software Package (DNASTAR, Madison, WI) and PHD (phd@EMB-Heidelberg) [13]. DNA and protein homology was examined with BLAST (<http://www.ncbi.nlm.nih.gov/blast/>) [14] and protein motifs with PROSITE (GCG version 8.0, Madison, WI). The Center for Biological Science Analysis (<http://www.cbs.dtu.dk/>) was used for NetStart, a translation start prediction program [15], SignalP V1.1, a signal sequence prediction program [16], and O-GlycBase V3.0, an O-glycosylation prediction program [17]. PSORT [18] was used to predict protein localization sites in eukaryotic cells.

2.4. Protein expression

The polymerase chain reaction with *Pfu*, a proof-reading polymerase (Stratagene), was used to subclone the *E. hellem* PTP gene from clone 1C1 using the following conditions: 94°C for

1 min, 25 cycles of 94°C for 1 min, 58°C for 1 min, 72°C for 3 min, followed by a 10 min extension at 72°C. Primers were designed to provide an *Eco*RI site at the 5' end (EhPTPstart4T1-*Eco*F: 5' CGGAATTCGCGAGTTCCGCTTTGCAGT 3') and a *Xho*I site at the 3' end (EhPTPEnd4T1-*Xho*R: 5' CCGCTCGAGCTAACATTGACAGCAGGAG 3'). After 25 cycles, the amplicon was purified using a Microcon 50 microconcentrator (Amicon, Beverly, MA), digested with *Eco*RI and *Xho*I in *Eco*RI buffer, enzymes were removed using EZ Micropure (Amicon, Beverly, MA) and the gene was ligated in frame into the *Eco*RI and *Xho*I sites of pGEX-4T1, a glutathione *S*-transferase (GST) expression vector (Pharmacia Biotech, Piscataway, NJ) creating pGEX-Eh₅₅. Recombinant protein was then purified from IPTG induced *E. coli* containing pGEX-Eh₅₅ using glutathione Sepharose 4B (Pharmacia Biotech) and analyzed by immunoblotting. Sequencing of the pGEX-Eh₅₅ insert confirmed that no misincorporations occurred in the cloning process.

3. Results

3.1. Purification of PTP of *E. Hellem*

The polar tubes of *E. hellem* demonstrated the same solubility properties as previously reported for *G. americanus*, that is, they were insoluble in 1% SDS and 9 M urea and dissociated in 2% DTT [3,9]. After pyridylethylation and reverse phase HPLC, the DTT-solubilized material demonstrated a major UV absorbing peak at a similar retention time as the major PTP peak of *G. americanus* (Fig. 1). The major reverse phase HPLC peak (i.e. PTP Eh₅₅) of *E. hellem* after SDS-PAGE and silver staining, migrated at 55 kDa (Fig. 1 lane B), compared to a migration at 43 kDa for *G. americanus* PTP (Fig. 1 lane A). Anti-PTP Eh₅₅ reacted to HPLC purified *E. hellem* PTP at 55 kDa by immunoblotting (Fig. 1 lane D) and also localized strongly to intrasporal polar filaments and extrasporal polar tubes of *E. hellem* (Fig. 2A–F) confirming the polar tube specificity of the antibody. Anti-PTP Eh₅₅ also cross-reacted by immunoblotting with *G. americanus* PTP (Fig. 1 lane C).

The PTP was relatively resistant to digestion by trypsin, endoproteinase Glu-C and cyanogen bromide in 70% formic acid. Reverse phase HPLC of the PTPs after each treatment revealed the presence of a major UV-absorbing peak with a similar retention time as the native protein suggesting a large resistant core in the protein (data not shown). Amino acid analysis of the purified PTP of *E. hellem* revealed a high proline content (10.9%), similar to that previously reported for *G. americanus* (18.2%) (Table 1). The protein also contained a high concentration of glycine, serine, glutamic acid/glutamine and threonine. A single N-terminal sequence (AVPLCSNTYDPSQ) was obtained for *E. hellem* PTP confirming the homogeneity of the purified protein.

3.2. Construction and screening of the genomic library

Following size fractionation of the partial *Sau*3AI digest of genomic DNA of *E. hellem*, and ligation into ZAP Express, the average insert size was 1.5 kb. After screening 160000 plaques with anti-PTP Eh₅₅, 14 antibody reactive genomic clones were identified. Plasmids 1D1 and 4B1, with inserts of 2.1 and 2.8 kb, were initially selected for sequencing. The 1D1 and 4B1 inserts were identical at the 5' ends, however, these inserts did not include the 5' end of the gene corresponding to the N-terminal sequence of the purified protein. Polymerase chain reaction of the other 12 antibody positive clones (using a forward primer 95 bp into the vector BKF: 5' ACAGGAAACAGCTATGACCTTG 3' and reverse primer 493 bp into the 1D1/4B1 insert PTP480r: 5' GAACCAACTGGCATGAGC 3') revealed three clones with additional 5' sequences. Clone 1C1 (2.3 kb insert) had identical sequences to clones 1D1 and 4B1 at its 3' end and included the sequences coding for the N-terminal sequence of the purified PTP Eh₅₅ protein at its 5' end.

The PTP Eh₅₅ gene consists of a 1362 bp sequence encoding 453 amino acids with no introns present (Fig. 3). Examination of the sequences with BLAST revealed no homology with other genes. The reading frame of this gene was determined by the frame of the β -galactosidase fusion protein in ZAP Express and was confirmed by the congruence of the translated amino acid sequence with the N-terminal sequence of HPLC purified PTP Eh₅₅. Three methionines are found in-frame upstream of the N-terminal sequence, with the furthest one predicted as the start sequence using NetStart [15]. This start site also translates into a putative signal sequence of 22 amino acids with a predicted cleavage site (SignalP V1.1) yielding the N-terminal sequence of the HPLC purified native protein [16] and predicted targeting to the endoplasmic reticulum (PSORT) [18]. After cleavage of the putative signal, the remainder of the coding sequence translates into a protein of 431 amino acids with a predicted molecular mass of 43 kDa. The amino acid analysis of the HPLC purified PTP Eh₅₅ and the predicted amino acid composition from the gene sequence are in excellent agreement (Table 1). Six alternating tandem repeats of 20 amino acids are present in this protein (Fig. 3). These repeat sequences form a protease resistant core as suggested by the protein digestion studies. Restriction enzyme digestion of genomic DNA is consistent with there being a single copy of the PTP Eh₅₅ gene in the haploid genome (data not shown).

3.3. Protein expression

The GST control plasmid (pGEX-4T1) and pGEX-Eh₅₅ expressed a protein of the expected size by SDS-PAGE and Coomassie staining: 29 kDa for GST and 84 kDa for pGEX-Eh₅₅ GST fusion protein. Anti-PTP Eh₅₅ reacted with the pGEX-Eh₅₅ GST fusion protein by immunoblotting and did not react with free GST which is consistent with the expressed protein being the same as the polar tube protein purified by reverse phase HPLC (data not shown).

4. Discussion

Our strategy for the purification of polar tube proteins was based on the ability of polar tubes to withstand dissociation by SDS and urea, but not to thiol reducing agents, e.g. DTT [1,2]. *E. hellem* PTP demonstrated the same solubility properties as reported for *G. americanus* [9]. Furthermore, the major PTP peak in the DTT-solubilized material demonstrated a similar retention time on reverse phase HPLC indicating similar hydrophobicity. This peak represented the majority of the DTT-solubilized protein by 214 nm absorption suggesting that PTP Eh₅₅ is the major component of the polar tube. By SDS-PAGE, the apparent subunit M_r of the purified PTP was 55 kDa, and it had a single N-terminus indicating homogeneity of the protein.

Polar tube specific anti-PTP Eh₅₅ was used to screen a genomic library of *E. hellem*. Given the small genome size of the microsporidia and that no introns have been reported in microsporidian genes to date, expression screening of genomic libraries proved to be a viable strategy for obtaining gene and protein sequences. Clone 1C1 contained the entire coding sequence of the mature protein including the sequence corresponding to the N-terminus of the HPLC purified PTP. When this predicted gene was ligated in frame into pGEX 4T-1, a GST fusion protein was produced that reacted by immunoblotting with the polar tube specific anti-PTP Eh₅₅ confirming the polar tube specificity of the protein coded by the gene.

The putative PTP gene is 1362 bp with the N-terminal sequence of the HPLC purified protein located 66 bp into the gene. Three methionines are found in-frame upstream of the N-terminal sequence, the furthest of which was predicted to be the start of this gene by translation start prediction programs such as NetStart [15]. This methionine, and not the

other methionines, results in a prediction of cleavage of the PTP gene to yield the correct N-terminus [16]. The 22 amino acid signal peptide is predictive by PSORT that PTP Eh₅₅ is processed through the endoplasmic reticulum [18]. Golgi and endoplasmic reticulum have been identified histologically in microsporidia [19]. The polar tube appears to form by the coalescence of vesicles that may be processed through this secretory pathway and the predicted signal peptide is in agreement with this morphologic data.

There is excellent correlation between the amino acid analysis of the HPLC purified PTP Eh₅₅ and the protein translation of the gene (Table 1) supporting the fact that they are the same protein. The major amino acids coded by the *E. hellem* PTP gene are proline (14.6%), glycine (13.0%), serine (11.8%) and threonine (10%). The protein lacks arginine, tryptophane and phenylalanine. This differs from a previously reported analysis of a 23 kDa PTP of *Ameson michaelis* in which the proline content was 3.5%, the arginine 3.5% and phenylalanine 3% [1]. Recently in an abstract Delbac et al. reported the identification of a putative PTP from the mammalian microsporidian, *E. cuniculi* that was also proline-rich (12.7%), and contained a putative 22 amino acid signal sequence at the N-terminus [20]. However, since no sequence data or Genbank accession number was provided it was not possible to compare these data with the *E. hellem* PTP gene we have described.

The PTP Eh₅₅ protein contains a central core of six tandem repeats of 20 amino acids which contain an internal motif of isoleucine-valine-tyrosine alternating with valine-isoleucine-serine in every other repeat (Fig. 3). The core is predominantly hydrophilic as each repeat contains 14 polar and six hydrophobic amino acids. This central repeating core lacks lysine/arginine, methionine, aspartic acid-proline bonds and glutamic acid, and consequently was resistant to digestion by trypsin, cyanogen bromide, formic acid and endoproteinase Glu-C, respectively. This accounts for the finding by HPLC of a major UV absorbing peak with a similar retention time as the native protein in post digestion samples. The protein has nine putative *N*-glycosylation sites (NXT/S), six of which are in the central repeating domain by PROSITE. Also, numerous putative *O*-glycosylation sites are predicted at the N-terminus of the protein, with virtually none occurring in the tandem repeat area [17]. In support of this prediction, polar tubes have been determined to have carbohydrate epitopes by histochemical methods [21].

The difference between the PTP molecular weight determined from the gene sequence (43 kDa) and from SDS-PAGE (55 kDa) may be the result of an aberrant migration in SDS-PAGE due to the high proline content of the protein or post-translational modification of the protein due to *N*-glycosylation or *O*-glycosylation. Utilizing the protean analysis program, PTP Eh₅₅ is predicted to have a high antigenicity index in the core and in the N-terminus. It is also predicted to be primarily a β -pleated sheet structure and have only three small α helix regions, all outside the central repeating domain with one predicted at the N-terminal signal sequence (protean and PHD programs [14]). Cysteine comprises 4.9% of the amino acids and is in the same position in each of the six repeats. It is likely that disulfide linkages between cysteine residues are important to the structure of the polar tube in that it solubilizes in the presence of thiol reducing agents such as dithiothreitol and 2-mercaptoethanol. A polyadenylation signal sequence, usually seen in higher eukaryotes (AATAAA), is found at the end of the coding region. An open reading frame encoding an unknown protein is present 54 bp upstream of the putative PTP start codon on the strand opposite the PTP coding strand. This is similar to the β -tubulin genes of *E. hellem* and *E. intestinalis* in which an open reading frame, also encoding an unknown protein, was observed 162 bp upstream of the start codon on the strand opposite the β -tubulin coding strand [22].

The high percentage of proline in the PTPs may contribute to the structural integrity of the polar tube. Proline, a hydrophobic imino acid, forms a fixed kink when present in a

polypeptide resulting in chain rigidity [23]. Some other proteins with high proline content include collagen, elastin and microfilarial sheath protein [23–25]. These proteins are known for their high tensile strength and/or elasticity and flexibility. Similar characteristics may be important for the polar tube to discharge and allow sporoplasm passage.

The polar filament which coils around the internal contents of the spore, is surrounded by a series of polaroplast membranes at its anterior end, and contains putative unpolymerized PTPs [2,26]. Upon spore activation, there is an increase in intrasporal osmotic pressure, the polar cap ruptures, and the polar filament everts from the spore in an explosive reaction forming the hollow polar tube that is two to three times the size of the coiled filament inside the spore [2,21,26]. The spore contents are pushed through this tube and emerge from the tip of the tube as a membrane-bound structure [26,27]. Since sporoplasm membrane remains in the empty spore coat, it is believed that the polaroplast membranes surrounding the polar filament in the spore form the new limiting membrane of the emerging nucleoplasm [1,21,27–29]. The polar tube increases in length as it is being everted, presumably by polymerization of PTPs at its growing tip [2,21,27]. A hypothesis for the formation of this tube involves rupture of the polar cap, eversion of the polar filament membrane at the anterior end, release of PTPs out of the polar filament and a simultaneous polymerization of the PTP on the outside of these membranes as they are being everted, thus creating the polar tube that increases in length at the tip. In this model, the lipophilic region of PTP Eh₅₅ would interact with the membrane and the hydrophilic regions would orient into an aqueous extrasporal environment. It is possible that a change in redox potential between the inside of the polar filament and outside of the spore at the growing tip triggers the formation of disulfide bonds in the PTPs favoring the polymerization process along these membranes. The tube would continue to elongate until the supply of unpolymerized PTP is exhausted. While PTP Eh₅₅ is clearly the major component of the polar tube it is possible that accessory proteins are important in the formation and functioning of this invasion organelle.

A single coiled polar filament is the structure which uniquely identifies microsporidian spores. Knowledge of the structure of polar tube proteins will further the understanding of the complex process of microsporidian invasion and may provide insights for therapeutic interventions. The finding of a highly antigenic central core may be important not only for polar tube structure, but may also serve as a target to detect antibodies in seroepidemiological surveys. Additional studies on the cloning and sequencing of polar tube genes are in progress.

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Abbreviations

AIDS	acquired immune deficiency syndrome
Anti-PTP Eh₅₅	polyclonal serum of rabbit immunized with purified 55 kDa polar tube protein of <i>Encephalitozoon hellem</i>
CSPD[®]	(disodium 3-(4-methoxy-1,2-dioxetane-3,2'-(5'-chloro)tricyclo[3.3.1.1] decan)-4-yl)phenyl phosphate)
DTT	dithiothreitol

GST	glutathione <i>S</i> -transferase
HPLC	high performance liquid chromatography
IPTG	isopropyl-1-thio- β -D-galactopyranoside
mAb	monoclonal antibody
PCR	polymerase chain reaction
PTP	polar tube protein
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis

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Fig. 1.

Reverse phase HPLC of the DTT-solubilized polar tube proteins of *Glugea americanus* and *Encephalitozoon hellem*. A Hewlett Packard Series II 1090 Liquid Chromatograph with a C8 column (Aquapore RP300, 220×2.1 mm, Applied Biosystems) was used (see Section 2[3,4]). Retention time for the major UV absorbing peaks (PTPs) was 30.6–31.9 min for *G. americanus* and 30.6–31.8 min for *E. hellem*. Insert: lanes A–B, silver stain of SDS-PAGE (10% polyacrylamide, reducing conditions [11]) of purified PTPs of *G. americanus* (lane A) and *E. hellem* (lane B); lanes C–D, immunoblot of Anti-PTP Eh₅₅ vs. the purified PTPs of *G. americanus* (lane C) and *E. hellem* (lane D). *G. americanus* migrated at 43 kDa and *E. hellem* at 55 kDa.

Fig. 2.

A–F: Transmission electron micrographs of a sporoblast, spores, and extruded polar tubes of *E. hellem* incubated with rabbit polyclonal anti-PTP Eh55 and immunolabeled with goat antirabbit IgG conjugated with 12 nm colloidal gold. A–D: bar = 0.5 μm ; (A) control, section through a spore revealing several portions of the polar filament (PF) as well as the thick endospore (EN). Note the lack of gold deposition. (B) late sporoblast indicated by the relatively thin EN coat, containing a large portion of the PF. Note the presence of gold deposition along the entire length of the filament. (C) spore containing several coils of the PF labeled with gold. (D) spore with a partially extruded PF. Note the deposition of gold along the entire length of the filament and a few gold particles in the posterior portion of the spore associated with some residual material. E–F: bar = 0.1 μm ; (E) portion of an extrasporal polar tube heavily decorated with gold particles. (F) section through an extrasporal polar tube revealing a portion of the lumen (arrow). Note the heavy decoration with gold particles.

[illegible]

Fig. 3. Nucleotide and amino acid sequence of PTP Eh₅₅ from *E. hellem*. The PTP Eh₅₅ gene is 1362 bp with a central core of six tandem repeats of 60 bp (20 amino acids). Shaded amino acid sequences correspond to the six repeats forming the central core of the protein. The amino acids corresponding to the N-terminal sequence of the HPLC purified protein are underlined. Three methionines occur upstream from the N-terminus of the protein with the furthest translating into a putative signal sequence of 22 amino acids. The protein also contained nine putative *N*-glycosylation sites (NXT/S), six of which were in the central core.

Table 1

Amino acid analysis of HPLC purified PTPs of *G. americanus* and *E. hellem* and amino acid composition of the protein translated from the PTP Eh₅₅ gene

Amino acid	% Of total amino acid (chemical)		From gene sequence
	<i>G. americanus</i>	<i>E. hellem</i>	<i>E. hellem</i>
Proline	18.2	10.9	14.6
Glycine	8.6	16.3	13.0
Serine	6.5	12.3	11.8
Threonine	8.4	9.8	10.0
Glutamine/glutamic acid	13.1 ^a	14.2 ^a	11.6 (9.5/2.1)
Valine	8.4	6.1	7.0
Isoleucine	3.7	3.6	6.7
Asparagine/aspartic acid	7.5 ^a	8.5 ^a	6.7 (5.1/1.6)
Cysteine	2.6 ^b	2.9 ^b	4.9
Alanine	4.4	5.1	4.4
Tyrosine	5.8	2.9	3.2
Leucine	2.8	1.9	2.6
Methionine	2.6	1.3	1.9
Lysine	3.5	2.6	1.4
Histidine	0.9	0.6	0.2
Arginine	2.3	0.9	0
Phenylalanine	0.7	50.1 ^c	0
Tryptophane	ND ^d	ND ^d	0

^a Amino acid analysis determines total glutamic acid/glutamine and total aspartic acid/asparagine.

^b Measured as pyridylethylcysteine.

^c Less than the limit of detection.

^d Not detectable by the method of analysis used.