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The putative chitin deacetylase of *Encephalitozoon cuniculi*: A surface protein implicated in microsporidian spore-wall formation

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Abstract

Microsporidia are fungal-like unicellular eukaryotes which develop as obligate intracellular parasites. They differentiate into resistant spores that are protected by a thick cell wall composed of glycoproteins and chitin. Despite an extensive description of the fibrillar structure of this wall, very little is known about its protein components and deposit mechanisms. In this study on the human pathogen *Encephalitozoon cuniculi*, we identify by mass spectrometry the target of polyclonal antibodies previously raised against a 33-kDa protein located at the outer face of the parasite plasma membrane. This 254-amino acid protein is encoded by the ECU11_0510 open reading frame and presents two isoforms of 33 and 55 kDa. Sequence analysis supports an assignment to the polysaccharide deacetylase family with a suspected chitin deacetylase activity (*Ec*CDA). As demonstrated by TEM studies, *Ec*CDA is present at the plasma membrane of the early stages of *E. cuniculi* life-cycle. At the sporoblast stage, the enzyme accumulates especially in paramural bodies which are convolutions of the plasma membrane opened to the wall. The identification of an *Ec*CDA homologue in the insect parasite *Antonospora locustae* (ex *Nosema locustae*) suggests a widespread distribution of this enzyme among Microsporidia. This characterization of a new microsporidian surface protein creates new perspectives to understand spore wall formation and spore resistance.

Keywords: Microsporidia; *Encephalitozoon cuniculi*; Chitin deacetylase

1. Introduction

The obligate intracellular Microsporidia are spore-forming amitochondriate unicellular eukaryotes that can parasitize a wide variety of animals ranging from protists to mammals. Some species, including *Encephalitozoon cuniculi*, are opportunist human pathogens [1] with a high seroprevalence in immunocompetent populations [2–4]. Phylogenetic analyses based on some conserved proteins suggest that Microsporidia are related to fungi [5,6]. Complete sequencing of the *E. cuniculi* genome [7] has also provided data consistent with a fungal origin of Microsporidia [8].

Microsporidian spores display an original invasion mechanism, involving a coiled structure (the polar tube) that can be quickly extruded to transfer the spore content or sporoplasm into a target cell. The parasite enters a proliferation phase or merogony and the resulting meronts then undergo a differentiation step (sporogony). They transform into sporonts (beginning of cell wall

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formation), then into sporoblasts (beginning of polar tube elaboration) and finally into mature spores protected by a thick wall. The microsporidian spore wall plays a major role in the protection against environmental stresses, allowing long-term survival of the parasite after its release from the host cell [9,10]. While the classical concept of the parasite penetration mode involves the polar tube acting as a needle–syringe system, microsporidian spores may also be internalized via attachment to the host plasma membrane then endocytosis [11]. If this occurs, the induced endocytosis process should depend on interactions with the wall.

The microsporidian spore wall usually comprises a proteinaceous outer electron-dense layer (exospore) and a chitinous inner electron-transparent layer (endospore) [12–14], which is of fibrillar nature and connected to the plasma membrane [13,15]. The stippled deposit of an electron-dense material on the outer side of the parasite plasma membrane is the main visible feature of the transition from merogony to sporogony [16,17]. This deposit turns into a continuous layer in young sporoblasts, and should be at the origin of the exospore. The electron-transparent endospore is built in later stages and thus appears as a cytological marker of the mature spore.

Few studies have been dedicated to the characterisation of spore wall proteins. Immunostaining with polyclonal antibodies raised against partially purified *E. cuniculi* proteins has revealed a 30-kDa antigen within the exospore and a 33-kDa one close to the spore plasma membrane [18]. Several monoclonal antibodies have also been reported to be specific to spore wall antigens [19–21]. To date, the knowledge of the primary structure of such antigens is limited to two exospore components with unknown function: SWP1 (50 kDa) in both *E. cuniculi* [22] and *Encephalitozoon intestinalis* [23], and SWP2 (150 kDa) in *E. intestinalis* only [23]. Here, we provide evidence for an *E. cuniculi* surface protein belonging to the polysaccharide deacetylase family that should be active in chitin deacetylation during all sporogonic stages.

2. Materials and methods

2.1. Microsporidian spore production and protein extraction

The *E. cuniculi* spores were produced on Madin–Darby Canine Kidney (MDCK) or Human Foreskin Fibroblast (HFF) cells as previously described [18].

Parasites (10^9–10^{10}) were disrupted in lysis buffer containing 100 mM DTT, 4% CHAPS and 0.2% SDS, by repeated cycle of freezing–thawing and sonication (Deltasonic 1320, 300 W, 28 kHz). Disrupted spores were then incubated in the extraction solution (7 M urea, 2 M thiourea, 100 mM DTT, 4% CHAPS, 0.2% SDS) for 6 h at room temperature. Soluble proteins were collected and stored at −20 °C.

2.2. Recombinant ECU11_0510 protein expression

The ECU11_0510 protein was expressed as a truncated form in fusion with Glutathione-S-transferase (GST) at the N-terminal and an 8xHis-tag at the C-terminal extremity. PCR primers were designed (5′-CGGAATTCTTTGTGGACGGTCCTGTC-3′ and 5′-CGCTCGAGTCCTATGCTCTCAACGCC-3′ containing an *Eco*RI and a *Xho*I restriction site, respectively) to amplify a 624-bp genomic DNA fragment corresponding to the amino acid region 32–239 of the 254-amino acid ECU11_0510 protein. PCR reactions were performed according to standard conditions with an annealing temperature of 55 °C, and the purified product was cloned in frame with GST into the prokaryotic expression vector pGEX-4T1 modified to provide a C-terminal 8xHis-tag after the *Xho*I site. The resulting recombinant plasmid was introduced in the *Escherichia coli* BL21+ strain. After induction with IPTG, bacterial proteins were extracted with Laemmli solution and analysed by SDS–PAGE.

2.3. Gel electrophoresis and Western blot analysis

Protein samples were analysed by SDS–PAGE on 12% polyacrylamide gels. For two-dimensional (2-D) gel electrophoresis, protein isoelectrofocalisation was performed along linear immobilized pH gradient strips of 7 and 17 cm (BioRad) using a rehydration buffer (7 M urea, 2 M thiourea, 4% CHAPS) supplemented with 2 mM tributyl phosphine (TBP) and 0.5% ampholytes (BioRad) with the IPGPhor apparatus (Amersham). Before standard SDS–PAGE on 12% polyacrylamide gel, strips were equilibrated with 50 mM Tris–HCl pH 8.8, 6 M urea, 30% glycerol, 2% SDS containing 100 mM DTT, and then 135 mM iodoacetamide. For mass spectrometry analysis, gels were fixed in a 7.5% acetic acid/30% ethanol solution, stained with Coomassie Blue (BioRad) and destained with 30% ethanol. For immunoblotting studies, proteins were transferred onto polyvinylidene difluoride (PVDF) membrane (Millipore). After blocking for 1 h in PBS-5% skim milk, membranes were incubated for 3 h with appropriate dilutions of polyclonal mouse antibodies in PBS 0.1% Triton X-100, treated with a 1:10000 dilution of alkaline phosphatase-conjugated goat anti-mouse IgG (H + L) (Promega) for 1 h at room temperature and revealed with NBT/BCIP (Promega).

2.4. Tryptic digestion for mass spectrometry analysis

Protein spots were manually excised from Coomassie blue-stained 2-D gels and transferred in 96-well-microtitration plates. Sample preparation was fully

automated (Mass Prep Station Multiprobe II, Micromass). Briefly, excised gels were washed with destaining solutions (25 mM NH_4HCO_3 for 15 min and 50% (v/v) acetonitrile containing 25 mM NH_4HCO_3 for 15 min). After dehydration with 100% acetonitrile and drying, proteins were reduced in 25 mM NH_4HCO_3, 10 mM DTT for 1 h at 37 °C, and alkylated in 25 mM NH_4HCO_3, 55 mM iodoacetamide for 30 min at 37 °C. After washing with destaining solutions and pure water, gels were shrunk again with 100% acetonitrile. Depending on protein amount, 2–3 μL of 0.1 μg/μL modified trypsin (Promega, sequencing grade) in 25 mM NH_4HCO_3 were added over the gel spots for 30 min. Seven μL of 25 mM NH_4HCO_3 were then added to cover the gel spots and digestion allowed to continue for 5 h at 37 °C.

2.5. MALDI-TOF-MS analysis and database search

For MALDI-TOF-MS analysis, 0.5 μL of trypsin peptide mixture was mixed with 0.5 μL of α-cyano-4-hydroxycinnamic acid at half saturation in 60% acetonitrile/0.1% (v/v) trifluoroacetic acid (TFA). The resulting solution was automatically spotted on the target plate and rinsed by 2 μL of 0.1% TFA for 30 s, liquid being then blown off by pressurized air. Peptide mixtures were analysed with a MALDI-TOF mass spectrometer (Autoflex, Bruker Daltonik) in reflector mode over a mass range of 0–4200 Da. For each sample, spectrum acquisition and annotation were done automatically. Spectra from 200 shots at several positions were combined to generate a peptide mass fingerprint for database searches. Spectra were calibrated using a mixture of angiotensin II (1046.54 Da), substance P (1347.74 Da), bombesin (1619.82 Da) and ACTH (18–39) (2465.20 Da) as an external standard. The *E. cuniculi* specific database was explored with an intranet version of Mascot 1.7. All peptide masses were assumed to be monoisotopic and $[M + H]^+$ (protonated molecular ions). Peptide modifications allowed during the search were cysteine carbamidomethylation, protein N-acetylation and methionine oxidation. The maximum number of missed cleavages was set to 1 and the mass tolerance to ±150 ppm.

2.6. Polyclonal antibody production

Antiserum to *E. coli* expressed recombinant protein was produced in BALB/c mice from crushed SDS–PAGE protein bands. Every two weeks, mice were injected intraperitoneally with samples homogenized with Freund's adjuvant (Sigma). Serum was collected 2 weeks after the last injection and stored at −20 °C.

2.7. Transmission electron microscopy immunolabelling

E. cuniculi-infected MDCK cells were pelleted, then fixed with 4% paraformaldehyde and 0.1% glutaraldehyde in 0.1 M phosphate buffer pH 7.2. After washing, the pellet was impregnated with a cryoprotective agent (25% glycerol, 5% DMSO in phosphate buffer) and frozen in pasty nitrogen before storage in liquid nitrogen. The 85 nm ultrathin sections were performed with the FCS system on the Leica UltraCut S ultramicrotome. The sections were picked on collodion-coated nickel grids and stored on 2.3 M sucrose before immunolabelling. After blocking for 30 min with 2% BSA in PBS, sections were incubated for 1 h30 with various dilutions of polyclonal antibodies, and then for 1 h with a 1:100 dilution of protein A or goat anti-mouse antibody conjugated with 5 nm colloidal gold particles (Sigma). After washing, the grids were stained and embedded with 0.5% aqueous uranyl acetate 1.6% methylcellulose for 10 min prior to examination with a JEOL 1200EX transmission electron microscope.

2.8. Sequence analysis

Physico-chemical parameters of the protein sequence were calculated with ProtParam (www.expasy.org). The SignalP program version 2 (www.cbs.dtu.dk/services/SignalP/), the TMPRED program (www.ch.embnet.org/software/TMPRED_form.html) and the PSORT II algorithm (www.psort.nibb.ac.jp/form2.html) were used to predict signal peptide, transmembrane helices and protein localisation, respectively. Consensus secondary structure prediction were performed on NPS@ Network Protein Sequence Analysis (http//npsa-pbil.ibcp.fr) [24] using the DSC, GOR4, PHD and SOPMA algorithms. Search for protein homologies and protein domains were done using the BLAST program (www.ncbi.nlm.nih.gov) and the Pfam database (www.sanger.ac.uk/Software/Pfam/search.shtml), respectively. Multiple sequence alignments were performed with Dialign program (bibiserv.techfak.uni-bielefeld.de/dialign/) and analyzed for amino acid identity and similarity with Boxshade (www.ch.embnet.org/sofware/BOX_form.html).

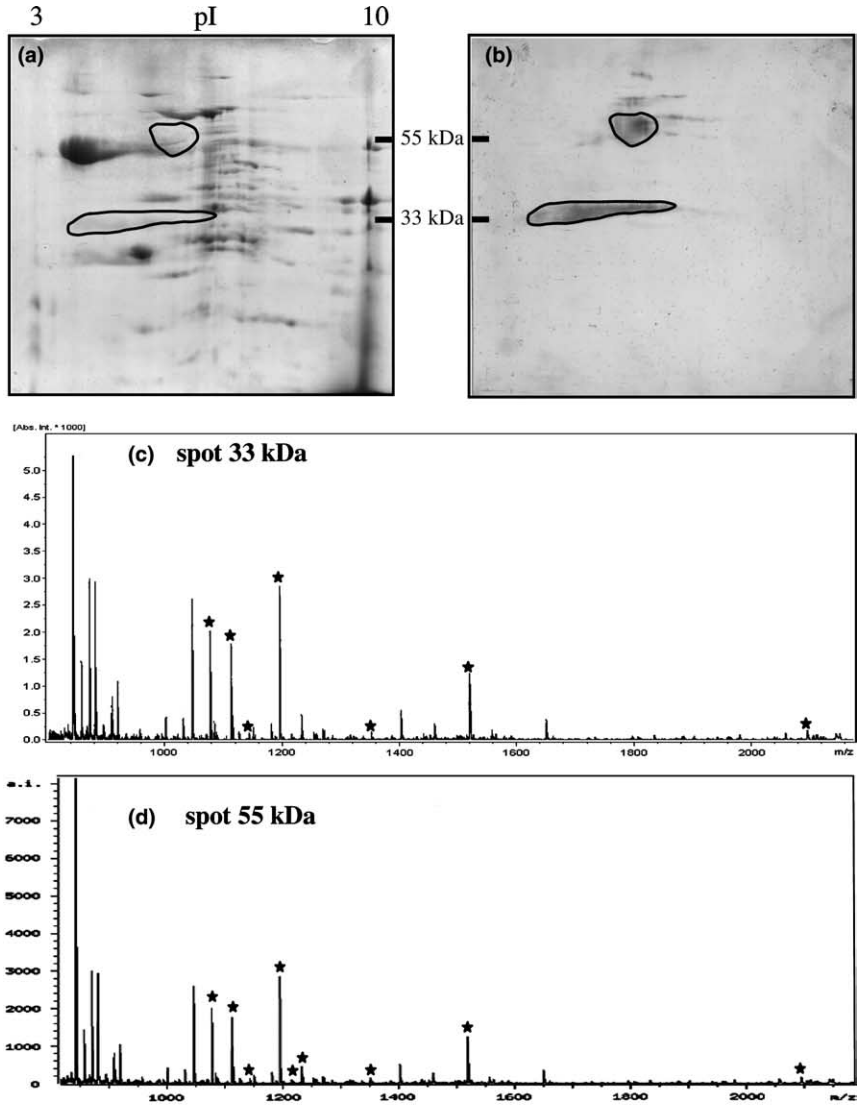
3. Results and discussion

Mouse polyclonal antibodies (PAb) were previously raised against several SDS–PAGE separated *E. cuniculi* spore proteins and analysed by Western blotting and indirect immunofluorescence [18]. One of these, directed against a 33-kDa protein band (anti-33 kDa PAb), showed a labelling close to the outer face of the parasite plasma membrane. This prompted us to identify the protein target of this anti-33 kDa PAb.

3.1. The anti-33 kDa PAb recognizes the ECU11_0510 protein

To get a better separation of the targeted protein, we performed 2-D electrophoresis of proteins extracted

from *E. cuniculi* spores (Fig. 1(a)). After Western blotting, the anti-33 kDa PAb highlighted two major regions at 33 and 55 kDa (Fig. 1(b)). MALDI-TOF mass spectrometry data obtained with the 33-kDa region (Fig. 1(c)) matched the ECU11_0510 protein (SWISS-PROT: Q8SU65) in the *E. cuniculi* proteome database deduced



(e)

Spot 33 kDa	Spot 55 kDa	Peptide m/z	MW (exp)	MW (calc)	Position	Sequence
-	+	1214.73	1213.72	1213.69	44-54	ILNTLDELGVK
+	+	1142.58	1141.58	1141.58	55-64	ATFSFTVNQK
+	+	1076.63	1075.63	1075.58	65-74	AVGNVGQLYR
-	+	1232.60	1231.59	1231.68	65-75	AVGNVGQLYRR
+	+	1350.74	1349.74	1349.72	75-86	RAVEEGHNVALR
+	+	1194.69	1193.69	1193.62	76-86	AVEEGHNVALR
+	+	1518.79	1517.78	1517.76	110-123	EIDTIDGLSGTEIR
+	+	2093.94	2092.93	2092.97	177-194	HHSFIILMHDGQEADTSR
+	+	1111.62	1110.61	1110.57	221-231	GAPGDPELSLR

Fig. 1. Migration on 2-D gel and identification by mass spectrometry of the anti-33 kDa PAb target. *E. cuniculi* spore proteins were separated by 2-D electrophoresis and either stained by Coomassie Blue (a) or subjected to Western blot immunodetection with a 1:4000 dilution of the anti-33 kDa PAb (b). Trypsin digests of the 33-kDa spot (c) and the 55-kDa spot (d) was analyzed by MALDI-TOF-MS. Seven (nine) experimental peptides for the 33-kDa spot (55-kDa one), labelled with stars in (c) (in (d)), matched the ECU11_0150 protein with a score of 60 (100) and sequence coverage of 29% (33.8%) (e). Only one miscleavage was observed. Differences between experimental and calculated molecular weights (MW) do not exceed 0.09 Da.

from genome sequencing [7] (Fig. 1(e)). Identical data were obtained with the 55-kDa spot (Fig. 1(d) and (e)). We produced a recombinant form of the *Ec*CDA protein which was slightly truncated to exclude predicted transmembrane helices (see below). The purified recombinant protein reacted with the anti-33 kDa PAb (Fig. 2) and was also used to produce specific PAb in BALB/c mice (anti-11_0510 PAb). These new antibodies exhibited a similar reaction pattern as the anti-33 kDa PAb either on the recombinant ECU11_0510 protein or on *E. cuniculi* extract (Fig. 2).

3.2. The ECU11_0510 protein is predicted to be a chitin deacetylase (Ec*CDA*)

As supported by genome data [7], the ECU11_0510 open reading frame is a 765-bp single-copy gene located on chromosome 11 which codes for a 254-amino acid protein (28.1 kDa, pI 4.56). As summarized in Fig. 3(a), the extreme N-terminal region displays the characteristics of a signal peptide with a predicted cleavage site between positions 15 (Gly) and 16 (Ser) corroborated by the TMPred prediction of a transmembrane helix (TMH) in the 1–18 region. An additional TMH should be located at the 234–253 C-terminal extremity in an outside–inside orientation, suggesting that the protein attached to the membrane via this TMH has a large extracellular domain. This is in accordance with the efficient recovery of ECU11_0510 (not shown) when using an improved membrane protein solubility mixture characterized by the amidosulfobetaine ASB14 detergent and high chaotrope molarity [25,26]. PSORT program also predicts an extracellular location including cell wall (66.7%, $k = 9/23$).

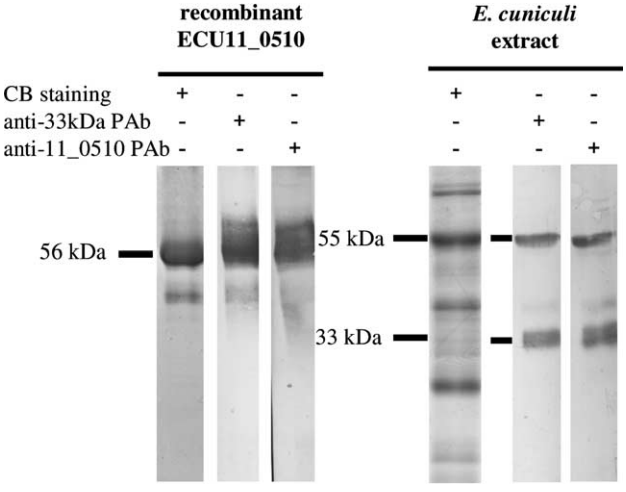


Fig. 2. Immunoblotting with anti-33 kDa PAb and anti-11_0510 Pab against both the recombinant ECU11_0510 protein and *E. cuniculi* extract. The 56-kDa recombinant form of the ECU11_0510 protein (fused in N-terminal with GST and in C-terminal with 8xHis tag) was injected into BALB/c mice to produce polyclonal antibodies (anti-11_0510 PAb). Both antibodies reacted in a similar way (CB: Coomassie Blue).

The ECU11_0510 protein contains the PfamA conserved domain 15022 representative of the polysaccharide deacetylase family (Fig. 3(b)). This family includes *Rhizobium* NodB (nodulation protein B), endoxylanases and fungal chitooligosaccharide deacetylases. The presence of chitin in the microsporidian spore wall has been unambiguously demonstrated [12,13]. Moreover, chitin is the sole polysaccharide that metabolism reconstruction predicts to be synthesized in *E. cuniculi* [7]. *N*-acetylglucosamine 6-P is converted into *N*-acetylglucosamine 1-P, then into UDP-*N*-acetylglucosamine via the activities of phosphoacetylglucosamine mutase and UDP-GlcNAc pyrophosphorylase. Chitin synthase carries out the polymerisation of *N*-acetylglucosamine into chitin chains. We thus assume that the ECU11_0510 protein may be a chitin deacetylase enzyme (*Ec*CDA) which deacetylates chitin into chitosan. Enzymatic assays with a purified recombinant protein that was expressed in *E. coli*, were unsuccessful. The recombinant protein may lack some post-translational modifications required for biological activity as it is the case for the yeast CDA2p which loses its activity after deglycosylation [27].

Interestingly, strong homologues of the chitosan pathway enzymes, including CDA, were identified in the partially sequenced genome of an insect Microsporidia, *Nosema locustae* renamed *Antonospora locustae* [28] ("*Nosema locustae* Genome Project, Marine Biological Laboratory at Woods Hole, funded by NSF award number 0135272", http://jbcp.mbl.edu/Nosema/index.html). The two microsporidian CDA sequences are of nearly identical size and exhibit 40% identity (Fig. 3(b)).

The presence of CDA in Microsporidia provides an additional support to the fungal origin of these intracellular parasites. Chitin deacetylases have been characterized in several fungi [29], including zygomycetes (*Mucor rouxii* [30,31]) and ascomycetes (*Colletotrichum lindemuthianum* [32], *Saccharomyces cerevisiae* [33,34]). These enzymes are monomeric glycoproteins ranging from 12 to 150 kDa in size, with a carbohydrate content ranging from 30% to 67% by weight in *Colletotrichum lindemuthianum* [32]. A well-defined role in cell-wall chitosan biosynthesis has been demonstrated for CDA secreted in the periplasm. In *M. rouxii*, chitin synthase first polymerizes chitin chains, then the *N*-acetamido bonds are hydrolysed by CDA [29]. In *S. cerevisiae*, two CDAs are required for the biosynthesis of a chitosan-containing ascospore wall [35], resistant to stress conditions such as glusulase or heat treatment [34,36]. In the microsporidian spore wall, chitosan could provide interactions with the other wall components and could thus account for the extreme rigidity and resistance of the parasite wall to chitinase treatments and to different stresses such as freezing, heating and extreme pH [9,10]. In some pathogen fungi, CDA is extracellular

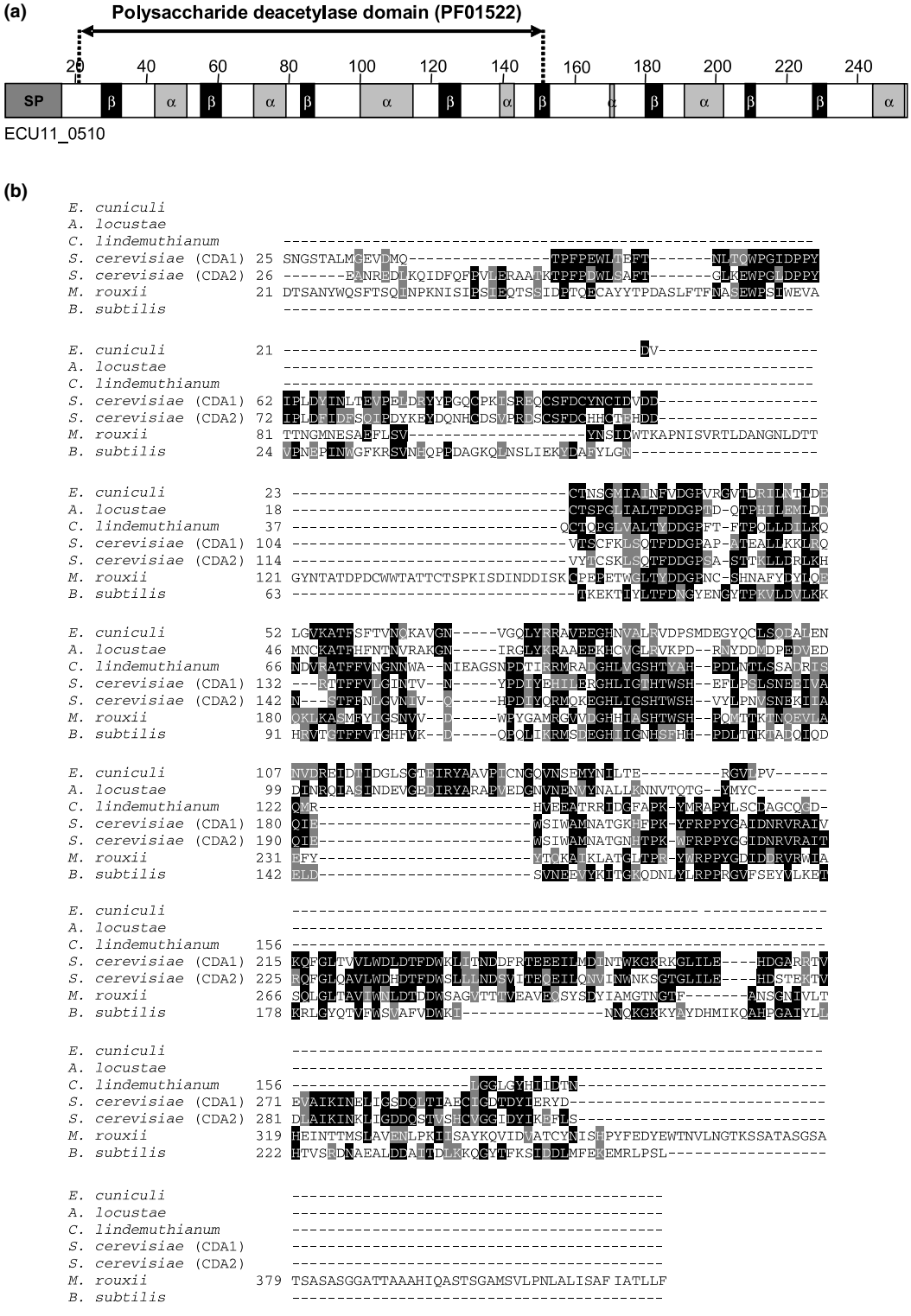


Fig. 3. Sequence analysis of the ECU11_0510 protein (a) and alignment with chitin deacetylase domains from other organisms (b). (a) The full-length protein (254 amino acids) is represented with different predicted elements: signal peptide (SP), secondary structure consensus with beta strand in black and alpha helix in grey. The ECU11_0510 polysaccharide deacetylase domain encompasses the position 21–150 and is included in the pfamA01522 domain (score = 11.1, E_{value} = 3.8e − 05). The predicted α/β class structure fits with the crystal three-dimensional model of *Bacillus subtilis* polysaccharide deacetylase (PDB: 1NY1). (b) Sequence alignment of chitin deacetylase domains from the mammalian Microsporidia *Encephalitozoon cuniculi* (ECU11_0510), the insect Microsporidia *Antonospora* (formerly *Nosema*) *locustae* (sequence from contig_159_1742:2506, http://jbpc.mbl.edu/Nosema/index.html, pfam: score = 56.4, E_{value} = 8.3e − 14), the fungi *Colletotrichum lindemuthianum*, *Saccharomyces cerevisiae*, *Mucor rouxii* and the bacterium *Bacillus subtilis*. Identical and similar residues are highlighted in black and grey respectively. The microsporidian domain is markedly reduced but presents a strong homology with the other proteins on its 60–70 first amino acids.

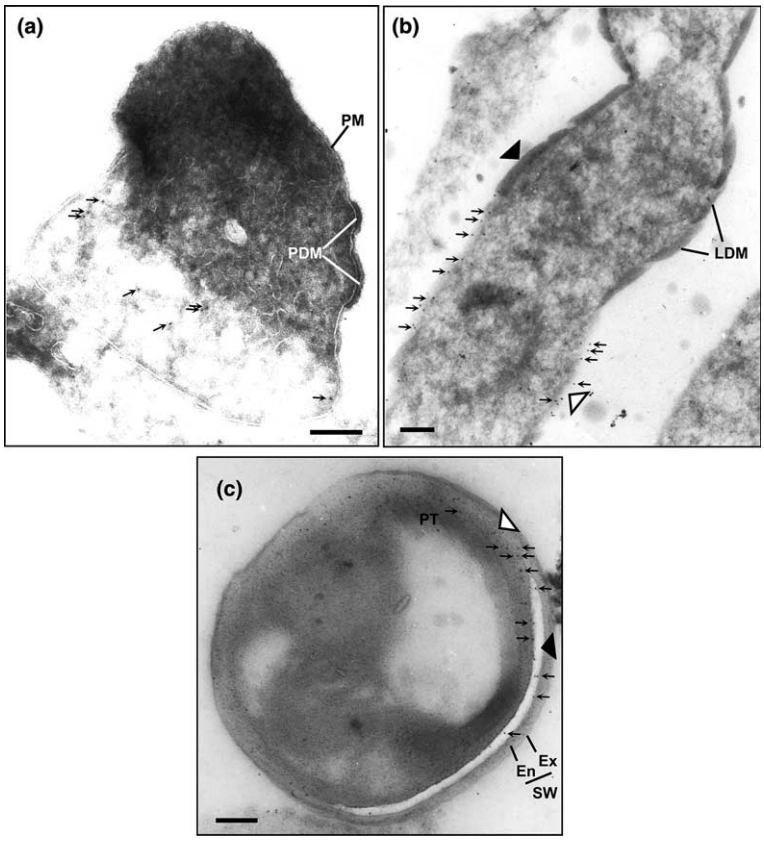


Fig. 4. Immunogold electron microscopy of cryosections of different *E. cuniculi* developmental stages using the anti-11_0510 PAb and secondary antibody conjugated with 5 nm colloidal gold (bars = 200 nm). The labelling (small arrows) is observed as early as the meront-to-sporont transition in the cytoplasm (a). In dividing sporonts (b), gold particles are found at the periphery of the cell. Finally, the antibodies react with the innermost part of the endospore of the mature spore (c). Black arrowheads highlight transversal sections of the spore wall, while the white ones show grazing section planes. PM: plasma membrane. PDM: patches of dense material. LDM: lamellar dense material. SW: spore wall. Ex: exospore. En: endospore. PT: polar tube.

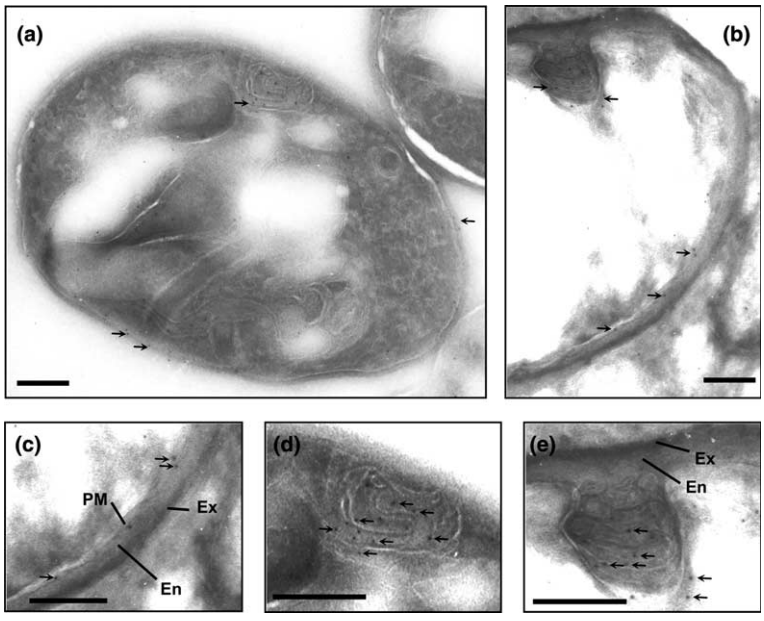


Fig. 5. Immunogold electron microscopy of cryosections of *E. cuniculi* sporoblasts using the anti-11_0510 PAb and secondary reagent labelled with 5 nm colloidal gold (bars = 200 nm). In sporoblasts (a,b), the labelling (small arrows) is associated with the constructing wall (c) and concentrates in peripheral structures (d,e). PM: plasma membrane. Ex: exospore. En: endospore.

and is thought to be implicated in the bypass of host-defence mechanisms during *C. lindemuthianum* plant invasion [32] or *Aspergillus fumagitus* mammalian infestation [37].

3.3. The Ec*CDA* protein presents two isoforms

In the *E. cuniculi* extract, both PAb recognized the two ECU11_0510 isoforms at 33 and 55 kDa (Fig. 2). Several fungi also exhibit multiple CDA glycoproteins: two, three, four and up to five isoforms were described in *Mucor rouxii* [31], *Metarhizium anisopliae* [38], *Rhizopus nigricans* [39] and *Uromyces viciae-fabae* [40], respectively. The *E. cuniculi* genome sequence predicts only few genes involved in protein glycosylation, all of them being related to O-mannosylation [7]. Sixteen serine and eleven threonine residues are potential O-glycosylation sites in *Ec*CDA. While O-mannoproteins were recently detected in the spore wall of *E. cuniculi* and in the SDS–PAGE 55-kDa region (Méténier, G., Mazet, M. and Taupin, V., personal communication), concanavalin-A labelling did not co-localize with the ECU11_0510 protein on 2-D gels (not shown).

3.4. Ec*CDA is located at the plasma membrane–endospore interface of the parasite*

Immunocytochemical experiments at the ultrastructural level with the anti-11_0510 PAb, revealed a slight gold labelling of the cytoplasm of stages representative of the meront-to-sporont transition (Fig. 4(a)). Such cells are indeed still tightly attached to the parasitophorous vacuole membrane by one side, and their engagement in early sporogony is marked by patches of dense material abutting on the plasma membrane. As sporogony progresses, the labelling tends to accumulate at the periphery of the parasitic cells. In longitudinal sections of dividing sporonts (Fig. 4(b)), gold particles are scattered just beneath the lamellar dense material deposit on the plasma membrane. In mature spores (Fig. 4(c)), gold particles are mainly seen at the plasma membrane–endospore interface. In sporoblasts, the *Ec*CDA is also present in the inner part of the cell wall tightly associated with the plasma membrane (Fig. 5(a)–(c)). This location is consistent with biochemical and sequence data, and suggests a role in cell-wall formation. Late sporogony is characterized by the thickening of the chitin-containing endospore [12], but the mechanism of chitin deposition is unknown. Since the *Ec*CDA enzyme is regularly distributed at the plasma membrane throughout sporogony, chitin may be produced all around the cell.

Late sporoblasts frequently harbour a membrane-rich structure (300×200–250 nm) which reacts with *Ec*CDA-specific PAb (Fig. 5(a), (b), (d) and (e)), and which opens to the wall. Gold particles are clearly associated with membranes of this structure that is filled with an electron-dense material similar to that of the endospore. Fig. 5(e) suggests that this material is flowing between the plasma membrane and the future exospore. In different Microsporidia such as *Pleistophora* sp., *Stempellia* sp. and *Ameson pulvis*, a similar membranous whorl termed ''scindosome'' or ''paramural body'' has been observed at the furrowing sites during division stage of the parasite [13,41].

Since the sporoblast decreases in size as its differentiation proceeds, the *E. cuniculi* paramural body could be interpreted as an endocytic recuperation of membrane/endospore material. Alternatively, chitin might be synthesized and deacetylated within the paramural body before being incorporated to the wall by an exocytosis-like process. As a result, this structure would be the equivalent of the fungal chitosome, an organelle which is specialized for chitin biosynthesis [42,43]. The chitosome hypothesis should be tested through further studies on the expression and location of microsporidian chitin synthase (ECU01_1390).

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