

Characterization and localization of an *Eimeria*-specific protein in *Eimeria maxima*

Raymond H. Fetterer · Ryan S. Schwarz ·
Katarzyna B. Miska · Mark C. Jenkins ·
Ruth C. Barfield · Charles Murphy

Received: 29 March 2013 / Accepted: 21 June 2013 / Published online: 3 July 2013
© Springer-Verlag Berlin Heidelberg (outside the USA) 2013

Abstract A recently completed analysis of *Eimeria maxima* transcriptome identified a gene with homology to sequences expressed by *E. tenella* and *E. acervulina* but lacking homology with other organisms including other apicomplexans. This gene, designated *Eimeria*-specific protein (ESP), codes for a protein with a predicted molecular weight of 19 kDa. The ESP gene was cloned and the recombinant protein expressed in bacteria and purified for preparation of specific antisera. Quantitative RT-PCR showed transcription of ESP was low in unsporulated oocysts and after 24 h of sporulation. However, transcription nearly doubled after 48 h of sporulation and reached its highest levels in sporozoites (SZ) and merozoites (MZ). The protein was detectable by Western blot in both sporulated oocysts and in SZ and MZ. Immuno-localization by light microscopy identified ESP in paired structures in the anterior of SZ and MZ. Immuno-localization by electron microscopy identified ESP in MZ rhoptries but no specific staining of any SZ structures was detected. In addition, localization studies on intestinal sections recovered from birds 120-h post-infection indicates that oocysts do not stain with anti-ESP but staining of microgametocytes and

developing oocysts was observed. The results indicate that ESP is associated with the rhoptry of *E. maxima* and that the protein may have functions in other developmental stages.

Introduction

Parasites of the genus *Eimeria* are the most ubiquitous and economically important parasite of chickens. Every chicken in a production system is considered to be infected with one or more *Eimeria* species and economic losses are estimated to be over 1 billion dollars annually (Allen and Fetterer 2002). Control of avian coccidiosis is currently accomplished by either medication of feed with anti-coccidial drugs or administration of live vaccines composed of low doses of *Eimeria* oocysts. The increasing incidence of drug-resistance and cost of live vaccines is prompting alternative control strategies, such as immunization of chickens with recombinant *Eimeria* proteins.

There have been several attempts to identify and characterize transcripts in developmental stages of *Eimeria maxima*, *Eimeria tenella*, and *Eimeria acervulina* (Wan et al. 1999; Ng et al. 2002; Li et al. 2003; Miska et al. 2004, 2008; Schwarz et al. 2010). In addition, a number of proteins primarily from developmental stages of *E. tenella* have been identified and characterized (Lal et al. 2009) but there remain a large number of genes or proteins that have been deposited in GenBank whose function is unknown. The genes or proteins that lack an apparent function and appear to be unique to *Eimeria* may be the most relevant ones to investigate. Unique genes and their products could prove useful as highly specific targets for recombinant vaccine development.

Recently, a transcriptome study of *E. maxima*, an important pathogenic species, identified a gene with homology to genes in *E. tenella* and *E. acervulina* (Schwarz et al. 2010). This gene denoted as *Eimeria*-specific protein (ESP) codes for a secreted protein with predicted molecular weight of 19 kDa. This gene lacks homology to known gene sequences from other Apicomplexa. Characterization of ESP is important because of its conservation in the three

R. H. Fetterer (✉) · M. C. Jenkins · R. C. Barfield
Animal Parasitic Diseases Laboratory, Beltsville Agricultural
Research Center, USDA/ARS, Beltsville, MD 20705, USA
e-mail: Raymond.Fetterer@ars.usda.gov

R. S. Schwarz
Bee Research Laboratory, Beltsville Agricultural Research Center,
USDA/ARS, Beltsville, MD 20705, USA

K. B. Miska
Animal Bioscience and Biotechnology Laboratory, Beltsville
Agricultural Research Center, USDA/ARS, Beltsville, MD 20705,
USA

C. Murphy
Soybean Genetics Improvement Laboratory, Beltsville
Agricultural Research Center, USDA/ARS, Beltsville, MD 20705,
USA

species and its potential as a target for a novel control of *Eimeria* parasites. In the current study, ESP from *E. maxima* was further characterized and found to be most highly expressed during the invasive stages of development and associated with the parasite's rhoptries.

Material and methods

Parasites and hosts

One-day-old Sexsals (Moyers Hatchery, Inc. USA) or broiler (Heritage strain; Longenecker's Hatchery, Inc. USA) chickens were housed in isolation and given food and water ad libitum. *E. maxima* (Tyson's strain) oocysts were maintained by passage through naïve Sexsals 3–4 weeks of age as previously described (Fetterer and Barfield 2003). All animal experiments were approved by the Beltsville Agricultural Research Center Animal Care and Use committee.

For studies of sporulation time-course, unsporulated oocysts collected from the feces of infected birds were incubated with sodium hypochlorite (6%) on ice for 10 min in twice the volume of the pellet with agitation every 2–3 min. Following bleach treatment, samples were diluted with water and the bleach removed by repeated centrifugation. Oocysts were suspended in PBS containing an antibiotic/antimycotic mixture (GIBCO, USA) and incubated under aeration at 41 °C. At the desired time interval (ranging from 0 to 72 h), an aliquot containing about 1×10^8 oocysts was removed from the incubation flask, centrifuged, and the pellet containing oocysts was resuspended in 1.0 ml of 40 mM Tris and stored at –70 °C.

Sporozoites (SZ) were prepared from sporulated oocysts (less than 30 days post-harvest) as previously described (Fetterer and Barfield 2003) and were purified by filtering through a cellulose filter pad (Fetterer et al. 2004; Fuller and McDougald 1990). Isolated SZ were prepared for microscopy as described below. For merozoites (MZ) collection, 10–20 broiler chicks (2–4 weeks of age) were inoculated with 5×10^5 sporulated oocysts per bird and sacrificed at 96-h post-infection. About 15 cm of the jejunum distal to Meckel's diverticulum was removed and MZ isolated as previously described (Schwarz et al. 2010). Isolated MZ were pelleted into HBSS and frozen at –70 °C or prepared for microscopy as described below.

Cloning and expression of recombinant *E. maxima* ESP

RNA extracted from *E. maxima* sporozoites using TRIzol reagent (Invitrogen, USA) as recommended was treated with two units of DNase then reverse transcribed (Access RT-PCR System; Promega, USA) using the following primers for *E. maxima* ESP: forward 5'-GTACGGATTTGCTTACAGTCAAG-3' and reverse 5'-GTCTTGACAGAAGACAGTTGTCTC-3' under the following conditions: 48 °C for 45 min; 94 °C for 2 min; 40 cycles

of 94 °C for 30 s; 53 °C for 60 s; 68 °C for 120 s; and 68 °C for 7 min. The 782-bp ESP amplicon was purified from the agarose gel using a gel extraction kit (Qiagen, USA), cloned into the pGEM-T easy vector system (Promega, USA) and recombinant plasmids were purified with QIAprep Spin Miniprep Kit (Qiagen, USA). Inserts were sequenced using the M13 vector priming sites for confirmation and this sequence for *E. maxima* ESP has been deposited to GenBank under the accession JN560940.

Another set of primers (ESP_1 forward: 5'-GCAGGATC CATGAAGGGAGCTATCC-3' and ESP_1 reverse: 5'-GCC GAATTCGTCTATTTACTGCTTGCTCCTCAG-3') were designed and used to amplify a 530-bp region of *E. maxima* ESP beginning at the predicted methionine start codon through to the predicted stop codon from the plasmid DNA described above under the following thermal cycling conditions: 95 °C 3 min; 30 cycles of 95 °C for 45 s; 60 °C for 30 s, and 72 °C for 60 s; and 72 °C for 7 min. These primers included BamHI and EcoRI restriction sites at the 5' and 3' ends, respectively. After gel electrophoresis the *E. maxima* ESP amplicon was purified (QIAquick Gel Extraction Kit; Qiagen, USA) and ligated into the expression vector system pTrcHis A (Invitrogen) for in frame ligation at the BamHI and EcoRI restriction sites, forming the recombinant *E. maxima* plasmid rEmESP-pTrcHis. Chemically competent DH5 α *Escherichia coli* were prepared according to standard protocol (Hanahan 1983) and transformed with rEmESP-pTrcHis. Appropriate target and orientation of inserts were confirmed by sequencing using the following flanking vector primers: sense 5'-CTGTACGACGATGACGATAAG-3'; antisense 5'-TCATCCGCCAAAACAGCCAAG-3'. *E. coli* BL21 (strain optimized for protein expression) (Novagen, USA) was transformed according to manufacturer protocol with the rEmESP-pTrcHis. PCR-confirmed recombinants were cultured at 37 °C in selective media (Luria-Bertani+100 μ g/ml ampicillin) to O.D.₆₀₀=0.6. The expression of rEmESP was induced with 1 mM isopropyl β -D-1-thiogalactopyranoside at 37 °C for 4 h. Cultures were centrifuged at 5,000 \times g for 10 min at 4 °C, the supernatant decanted and the pellet resuspended at 1:20 of original culture concentration (OC) in native binding buffer (20-mM sodium phosphate; 500-mM sodium chloride; pH 7.8) followed with the addition of phenylmethylsulfonyl fluoride protease inhibitor at 1:10 of the suspension volume and frozen at –70 °C. Three rounds of freeze-thaw using an ethanol dry-ice bath and 37 °C water bath were performed to lyse the cells, which were then treated with DNase and RNase (1:10,000 OC) for 30 min at room temperature and centrifuged for 10 min at 5,000 \times g. The soluble fraction containing rEmESP was collected and purified using immobilized metal affinity chromatography on a nickel-nitrilotriacetic acid agarose column (Qiagen, USA) according to manufacturer guidelines, visualized using SDS-PAGE and quantified using bicinchoninic acid (BCA) analysis (Thermo Scientific, USA).

Quantitative RT-PCR analysis

Total RNA was isolated from *E. maxima* as above then treated with 1 U/ μ g of RQ1 RNase-free DNase (Promega, USA) prior to first-strand cDNA synthesis using the Advantage[®] RT-for-PCR Kit with random hexamers for priming (Clontech Laboratories, USA). To compare the relative expression of ESP during *E. maxima* development, 1 μ g of cDNA template was combined with 150 nM forward and reverse primer and 1x EvaGreen MasterMix (Biotium Inc., USA) in 25- μ l total volume then real-time PCR amplified on the Mx3000P (Stratagene, USA) using the following cycling conditions: 95 °C for 10 min; 40 cycles of 95 °C for 30 s; 57 °C for 30 s; 72 °C for 40 s; and a dissociation curve generated by ramping from 55 to 95 °C.

The transcript levels of two candidate housekeeping genes, 18S rRNA and glyceraldehyde-3-phosphate dehydrogenase (GAPDH), were empirically tested for their suitability as normalizing standards in RNA isolated from the developmental stages of *E. maxima*. Primers designed to amplify the *E. maxima* GAPDH gene proved to be differentially expressed while primers targeting the small subunit rRNA (18S rRNA) gene (forward 5'-CGGTGAACTGCGAATGGCTCA-3' and reverse 5'-GCCTTCCTTAGATGTGGTAGCC-3') were consistently expressed and thus used as the reference gene to calculate the relative expression level of ESP as: $(C_T^{\text{reference}} - C_T^{\text{target}})$. The mean of three technical replicates were calculated and the dataset was transformed to a positive scale in a linear fashion using the following: $X = Y + K + 1$ where Y is the relative expression level for a sample, K (22.37) is the lowest Y value from the dataset and 1 to plot the lowest value in the dataset at 1 (Schwarz and Evans 2013). A 130-bp region of *E. maxima* ESP spanning an intron was amplified using forward: 5'-GCTGC TCCTGAGGACATCAG-3' and reverse: 5'-CCTTCTCCA ACTCCTCAGTGCT-3'. These primers were designed to span an intron to avoid amplifying any contaminating gDNA that may have been present following the RNA extraction. Values were expressed as mean \pm 1 SEM.

Antibody production

Antisera production was performed by a commercial company (Pacific Immunology, USA; Kim et al. 2010). In brief, antisera was generated by a primary immunization with affinity-purified recombinant ESP (rESP) emulsified in Freund's Complete Adjuvant, followed by two booster immunizations with rESP in Freund's Incomplete Adjuvant. After the final immunization, sera were collected from rabbits and frozen (-70 °C) in 1-ml aliquots.

Electrophoresis and Western blot analysis

Soluble extracts of *E. maxima* oocysts were prepared in 40-mM Tris, pH 8.0 containing a cocktail of protease inhibitor DNase and RNase as previously described (Fetterer et al. 2004).

Samples were incubated for 10 min at 37 °C and then frozen at -70 °C. Concentrations of soluble proteins were measured by BCA assay (Thermo Scientific, USA) with bovine serum albumin as the standard. Samples were analyzed by polyacrylamide gel electrophoresis using 1-mm thick gradient gels (8–9 cm, 4–12 % Bis-Tris, Invitrogen, USA) as described (Fetterer and Barfield 2003). All samples were reduced by addition of 10-mM dithiothreitol. Extracts of sporulated oocysts, SZ and MZ were loaded onto gels at about 10 μ g of protein per lane. Western blot analysis was performed using the method previously described (Fetterer and Barfield 2003). Rabbit primary antibody was used in dilutions from 1:500 to 1:1,000. Secondary antibody was goat anti-rabbit conjugated to HRP (20 ng/ml; Thermo Scientific, USA). Chemiluminescence of blots was visualized with a digital camera after exposure to Luminol (SuperSignal WestDuraExtend, Thermo Scientific, USA). Western blots were quantified with a gel analysis system (Labworks, UVP, USA).

Antibody localization

For indirect fluorescent antibody staining (IFA) of *E. maxima*, SZ or MZ were recovered from infected chickens and purified as described above. Parasites were air dried or fixed with methanol or acetone, processed as previously described (Fetterer et al. 2004), and visualized at \times 400 magnification with a Zeiss compound microscope. A 1:250 to 1:1,000 dilution of rabbit anti-ESP was used as the primary antibody and 1:100 dilution of FITC-labeled anti-rabbit IgG (Pierce) as a secondary antibody. For antibody localization in infected intestine, tissue was isolated and fixed in 10 % formalin, and 10- μ m paraffin sections were prepared (Histoserv Inc., USA). Sections were stained with 1:1000 dilution of rabbit anti-ESP as the primary antibody using Dako (Denmark) Envision with AEC as the substrate chromogen following manufacturer's recommendation.

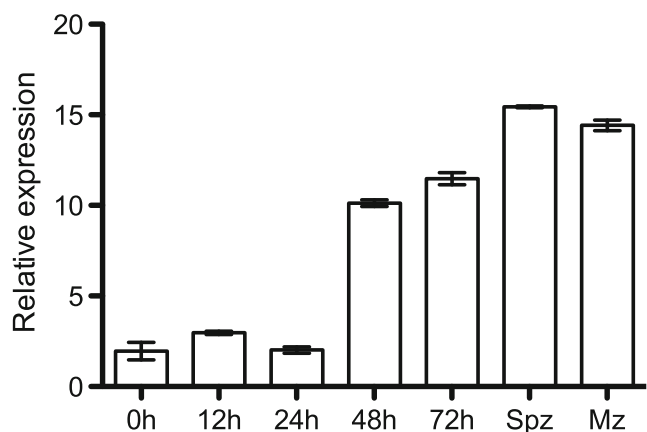


Fig. 1 The relative expression of ESP transcripts from developmental stages of *E. maxima*. The relative expression of ESP from *E. maxima* unsporulated oocysts (0 h), sporulating oocysts (12–48 h), sporulated oocysts (72 h), sporozoites (*Spz*) and merozoites (*Mz*) were plotted after being normalized to 18S rRNA. Values are means \pm SEM; $n=3$ technical replicates

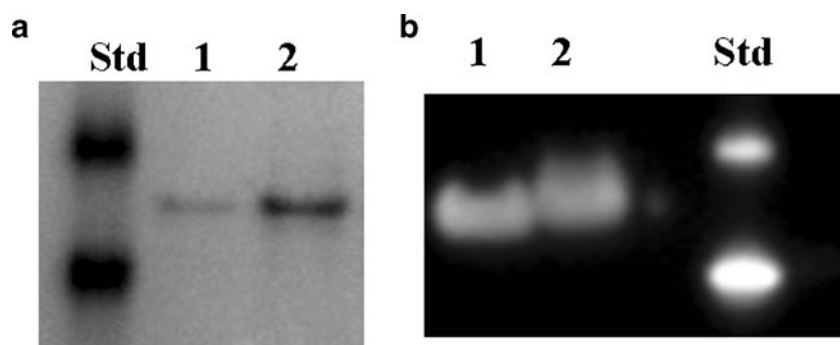


Fig. 2 Gel analysis of recombinant and native ESP. **a** Gel electrophoresis of recombinant ESP (rESP). About 1 (*lane 1*) or 5 μ g (*lane 2*) was loaded in each lane. Gel was stained with Coomassie

blue. Standards (*std*) have M_r of 21 and 27 kDa. **b** Western blot analysis of *E. maxima* MZ *lane 1* or SZ *lane 2*. About 3.0- μ g were loaded per lane. Standards (*Std*) have M_r of 20 and 30 kDa

For electron microscopy, SZ and MZ isolated as above were fixed for 5 min at room temperature by immersion into 4.0 % paraformaldehyde/0.5 % glutaraldehyde/0.05-M Na-Cacodylate buffer at pH 7.0. This was followed by washing in a 0.1-M Na-Cacodylate buffer rinse, six times over 1 h, dehydrated in an ethanol series and infiltrated with hard-grade acrylic resin (LR White, London Resin Company, UK) and cured at 55 °C for 24 h. The samples were centrifuged at about 1,000 \times *g* for 10 min between each solution change. Ninety-nanometer silver–gold sections of the tissue were obtained on a Reichert/AO Ultracut microtome with a Diatome diamond knife and collected onto 200 mesh formvar coated Ni grids. The grids were floated with the tissue section facing down on drops of PBS containing 0.1-M glycine and 1 % BSA for 10 min, washed with PBS, floated on drops of PBS–2 % nonfat dry milk –0.1 % Tween 20 (Tw20), and then floated on drops containing a 1:250 to 1:1,000 dilution of rabbit antiserum in PBS–NFD–Tw20. Rabbit antiserum to rESP was used in the primary labeling step. The grids were incubated for 2 h at room temperature, washed three times with PBS–NFD–Tw20, and floated for 1 h at room temperature on drops of a 1:50 dilution of gold

particle (10 nm diameter)-labeled goat anti-rabbit IgG (H–L chain specific, Sigma, USA). The grids were washed three times with PBS–Tw20, twice deionized H₂O, air dried, stained with 5 % uranyl acetate for 30 min, and examined with a Hitachi H7000 electron microscope.

Results

Quantitative analysis of ESP transcripts in *E. maxima*

The transcript expression profile of ESP in developmental stages of *E. maxima* was determined by quantitative RT-PCR. These data were normalized to the level of 18S rRNA from the same template sample and plotted as relative expression (Fig. 1) as described. ESP was transcribed at relatively low levels in oocysts at 0 h, 12 h, and 24-h sporulation with mean relative expression = 1.943 \pm 0.489, 2.950 \pm 0.085, and 2.003 \pm 0.171, respectively. At 48 h, ESP relative expression levels approximately doubled (Fig. 1) and reached cycling threshold \sim 8 cycles earlier, increasing mean relative expression levels to 10.107 \pm 0.177 in 48-h sporulated oocysts. ESP

Fig. 3 Immuno-localization by IFA of anti-ESP in *E. maxima* SZ (**a**) and MZ (**b**). The *arrows* indicate location of anti-ESP stained with fluorescently tagged anti-rabbit IgG

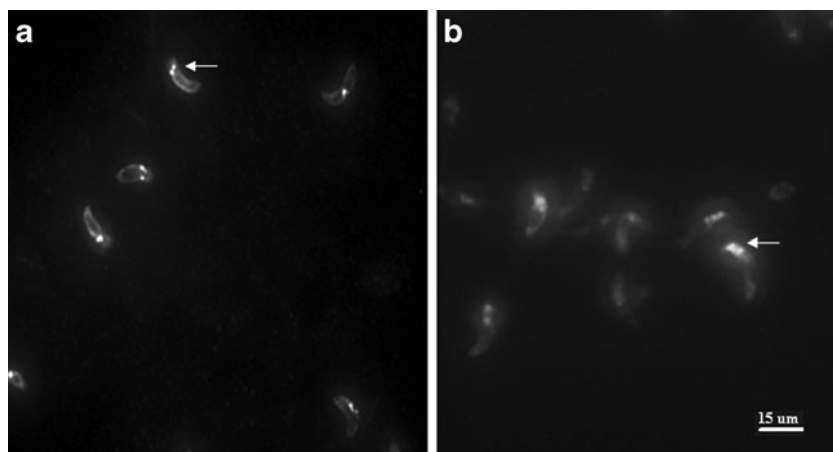
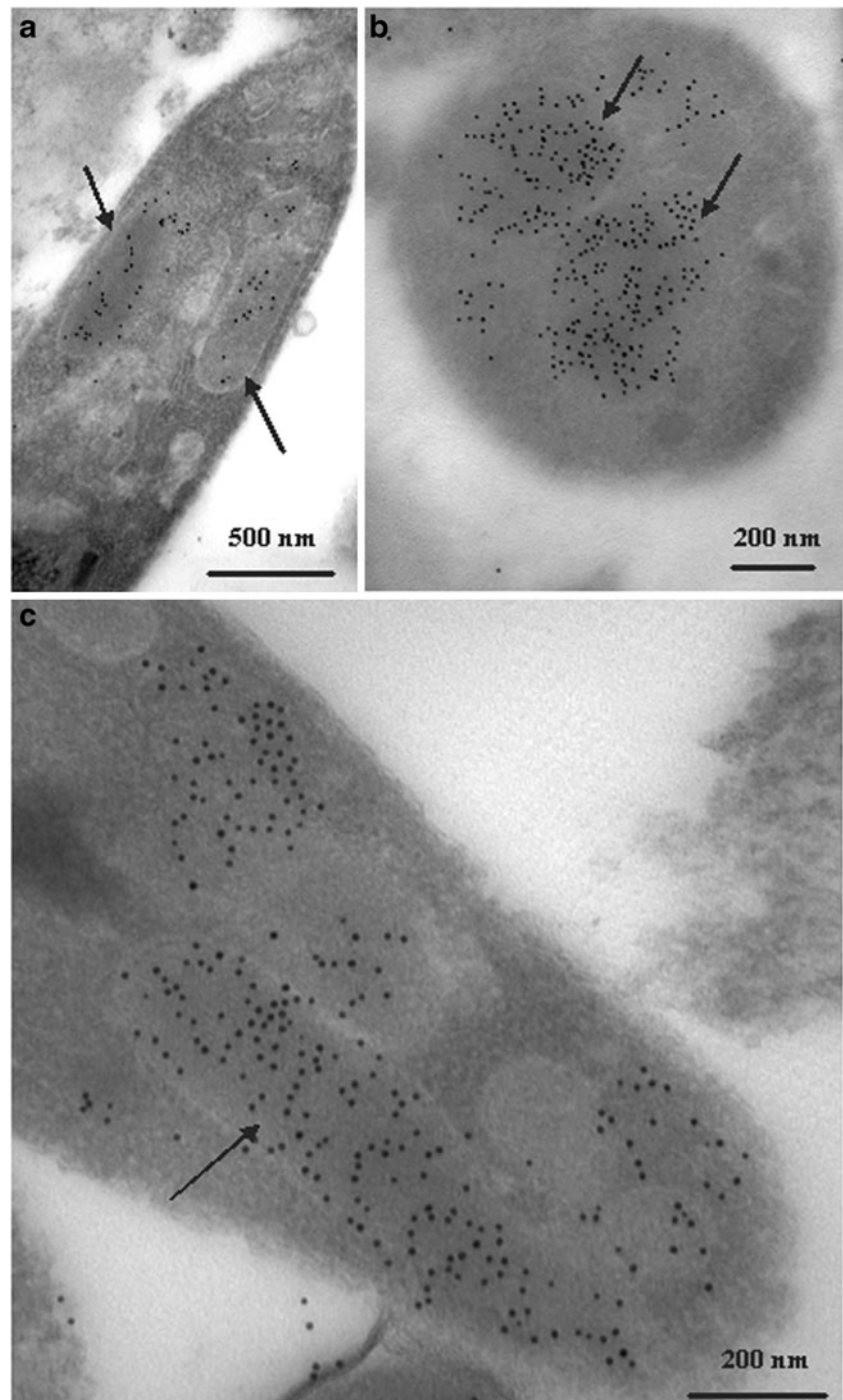


Fig. 4 Ultrastructural localization of anti-ESP in *E. maxima* MZ. Arrows indicate dark grains that visualize the bound antibody



transcription maintained similar levels through sporulation at 72 h (11.460 ± 0.338) then reached highest levels of relative transcription in SZ (15.430 ± 0.053) and MZ (14.403 ± 0.295).

Western blot analysis of ESP in developmental stages

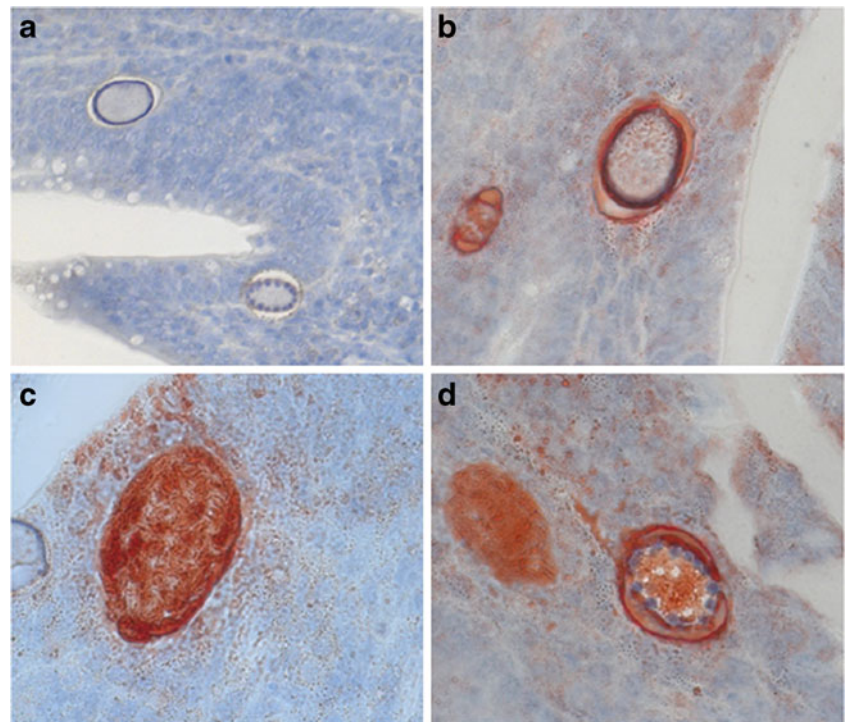
The purified rESP analyzed by gel electrophoresis had a relative molecular weight (Mr) of 24 kDa (Fig. 2a) and stained specifically with the anti-ESP by Western blot (not shown). The theoretical molecular weight (MW) for the

entire recombinant predicted translation is 23 kDa while the predicted MW for the native protein is 19 kDa. Western blot analysis of SZ and MZ extracts indicate a single band with Mr of about 23 to 24 kDa recognized by anti-ESP (Fig. 2b)

Antibody staining

Epifluorescence microscopy of IFA staining of *E. maxima* SZ indicated that ESP was localized to paired structures in the parasite's anterior (Fig. 3a). Staining of MZ was also localized to

Fig. 5 Staining of sections of tissue from chicken mid intestine with anti-ESP 120 h after infection with 25,000 oocysts. Positive staining is indicated by *dark red* color. **a** Staining with pre-bleed sera. **b** Oocysts. **c** Microgametocytes. **d** Developing oocysts showing wall-forming bodies (*blue* stain)



structures in the parasite's anterior (Fig. 3b). Pre-bleed sera did not react with either SZ or MZ (not shown). Ultrastructural studies demonstrated specific staining of anti-ESP in paired structures in the anterior of the MZ consistent with the morphology of the rhoptries (Fig. 4). In approximate longitudinal sections, the structures appeared elongated (Fig. 4a and c) while in an approximated cross section, the structures are circular (Fig. 4b). In some sections, staining could be observed in membranous structures that were not obviously rhoptries (Fig. 4c). Staining of structures in *E. maxima* SZ was not observed (not shown).

Staining of intestine 120 h after infection of birds with *E. maxima* oocysts demonstrated limited staining of oocysts with staining restricted to outer parasitophorous vacuole (PV) membranes (Fig. 5b). In developing oocysts, weak staining was observed in cytoplasm and PV membranes. Wall-forming bodies were not stained (Fig 5d). General staining of microgametocytes was observed (Fig. 5c) however, staining of macrogametes was not observed (not shown).

Discussion

Previous studies of the *E. maxima* transcriptome reported a predicted protein, denominated ESP, which is abundantly expressed in three *Eimeria* species, is not homologous to other proteins outside the genus *Eimeria*, and is without known function (Schwarz et al. 2010). The current results extend the

knowledge of ESP and indicate it is differentially transcribed throughout development and the protein is associated with both the rhoptries, the PV membranes surrounding the developing oocysts and the microgametes.

Three distinct organelles comprise the apical complex of Apicomplexan parasites: rhoptries, micronemes, and dense granules. All of these organelles are unique to Apicomplexa and play critical roles in mediating host cell invasion (Katz et al. 2006). As a result, proteins produced by these organelles are frequently targeted for vaccine development as a way of minimizing cross-reactivity with host epitopes and for the potential of blocking critical components to host cell invasion. Unlike microneme proteins, which are highly conserved across Apicomplexa (Tomley and Soldati 2001), putative rhoptry proteins are very divergent across taxa (ex. *Plasmodium* vs. *Toxoplasma* vs. *Eimeria*) and may reflect highly specialized coevolution of these parasite proteins with their specific host targets (Boothroyd and Dubremetz 2008). That ESP is unique to *Eimeria* species is most congruous with our current understanding of rhoptry proteins but not with microneme proteins.

Both light and electron microscopy localized anti-ESP antibody to paired organelles in the anterior of MZ consistent with the structure of the *Eimeria* rhoptries (Jenkins et al. 1990). The localization of ESP to the rhoptries and potentially other organelles at the apical end of SZ and MZ was the most striking observation and supports a functional role for ESP in mediating host cell interaction. Ultrastructural studies suggest the protein is distributed throughout the rhoptries rather than to specific regions (bulb or neck) as reported for some rhoptry proteins in the

related apicomplexan parasites *Plasmodium falciparum* and *Toxoplasma gondii* (Rodger et al. 1988; Bradley et al. 2005). In contrast to our results using MZ, no specific staining of SZ by anti-ESP was observed by electron microscopy although light microscopy clearly showed staining in the anterior of the SZ. The reason for this is not apparent. Although it could not be detected by electron microscopy within the SZ and ESP transcripts were found to be highly expressed in SZ by quantitative RT-PCR. Additionally, ESP was detected by Western blot in fully sporulated oocysts, (which contain SZ) as well as isolated SZ.

Although ESP is clearly associated with the rhoptries of SZ and MZ, it was also observed in PV surrounding oocysts and in microgametocytes embedded in host tissue, suggesting important roles beyond initial host cell invasion, possibly maintenance of intracellular stability within the host cell. It is known that some rhoptry proteins from Apicomplexa are secreted during host cell invasion and associate with the PV and PV membrane that forms around the parasite embedded in a host cell (reviewed in Boothroyd and Dubremetz 2008). We have shown that transcription of ESP is active to varying degrees throughout parasite development, congruent with our detection of ESP protein to particular regions of parasites at multiple development stages. Similar to our findings with ESP, previous studies of SZ proteins from *Eimeria*, such as the microneme protein MIC2, have increased transcription prior to completion of sporulation and formation of SZ within the oocyst (Sasai et al. 2008; Ryan et al. 2000). A similar pattern of expression would be expected for rhoptry proteins since they are also part of the apical complex. Taken together, these data are consistent with a functional role for ESP as a protein that mediates interaction with the host environment and may also serve as a structural protein of PV membranes and microgametes.

Proteins specific to the rhoptries of the Apicomplexans *T. gondii*, *Neospora caninum*, and *P. falciparum* have been characterized and range in molecular weight from 40–240 kDa (Bradley et al. 2005; Bradley and Sibley 2007; Marugan-Hernandez et al. 2011). Recently, the rhoptry proteome from *E. tenella* has been characterized (Oakes et al. 2013). A large number of proteins were identified in the *E. tenella* rhoptry, many with homology to proteins from *T. gondii* and *N. caninum*. Since they did not screen their data against an *Eimeria* panel of proteins, ESP was not identified because there are no homologues for ESP present in Apicomplexa clades (e.g., *Toxoplasma* or *Neospora*) outside of *Eimeria* as confirmed in Schwarz et al. 2010. Thus, the absence of ESP from these data is misleading. A recently published expressed sequence tag collection produced from *E. tenella* (Amiruddin et al. 2012) was screened prior to the protein accession of ESP resulting from this study (AEN84770) and therefore did not identify ESP in their data at the time. However, we reexamined their data and indeed found ESP to be present in their “Predicted Secreted Proteins” data file from *E. tenella* (sequence ID cn1070_Etm102F05) with a BLASTx e-value of 3.00E-63

match to accession AEN84770 (*E. maxima* ESP). Their analysis of the ESP sequence confirmed the presence of a signal peptide and the lack of transmembrane or GPI anchor regions, consistent with the initial characterization of ESP by Schwarz et al. 2010. In conclusion, this supports that ESP is a secreted protein unique to *Eimeria* species that localizes to the rhoptry organelles and PV where it most likely mediates host cell interaction.

Acknowledgments We are indebted to D. Hawkins-Cooper and C. Lowe for expert technical assistance.

References

- Allen PC, Fetterer RH (2002) Recent advances in biology and immunobiology of *Eimeria* species and the diagnosis and control of infection with these coccidian parasites of poultry. *Clin Microbiol Rev* 15:58–65
- Amiruddin N, Lee X-W, Blake DP, Suzuki Y, Tay Y-L, Lim L-S, Tomley FM, Watanabe J, Sugimoto C, Wan K-L (2012) Characterisation of full-length cDNA sequences provides insights into the *Eimeria tenella* transcriptome. *BMC Genomics* 13:21
- Boothroyd JC, Dubremetz JF (2008) Kiss and spit: the dual roles of *Toxoplasma rhoptries*. *Nat Rev Microbiol* 6:79–88
- Bradley PJ, Sibley DS (2007) Rhoptries: an arsenal of secreted virulence factors. *Curr Opin Microbiol* 10:582–587
- Bradley PJ, Ward C, Cheng S, Alexander DL, Collier S, Coombs G, Dunn DJ, Ferguson DJ, Sanderson SJ, Wastling JM, Boothroyd JC (2005) Proteomic analysis of rhoptry organelles reveals many novel constituents for host–parasite interaction in *Toxoplasma gondii*. *J Biochem* 280:34245–34258
- Fetterer RH, Barfield RC (2003) Characterization of a developmentally regulated oocyst protein from *Eimeria tenella*. *J Parasitol* 89:553–564
- Fetterer RH, Miska KB, Jenkins MC, Barfield RC (2004) A Conserved 19 kDa *Eimeria tenella* antigen is a profilin-like protein. *J Parasitol* 90:1321–1328
- Fuller AL, McDougald LR (1990) Reduction in cell entry of *Eimeria tenella* (Coccidia) sporozoites by protease inhibitors and partial characterization of proteolytic activity associated with intact sporozoites and merozoites. *J Parasitol* 76:464–467
- Hanahan D (1983) Studies on transformation of *Escherichia coli* with plasmids. *J Mol Biol* 166:557–580
- Jenkins MC, Lillehoj HS, Barta JR, Danforth HD, Strohlein DA (1990) *Eimeria acervulina*: cloning of a cDNA encoding and immunogenic region of several related merozoite surface and rhoptry proteins. *Exp Parasitol* 70:353–356
- Katz LM, Black CG, Proellocks NI, Coppel RL (2006) Plasmodium rhoptries: how things went pear-shaped. *Trends Parasitol* 22:269–276
- Kim S, Miska KB, Jenkins MC, Fetterer RH, Cox CM, Suard LH, Dalloul DA (2010) Molecular cloning and functional characterization of the avian macrophage migration inhibitory factor (MIF). *Dev Comp Immunol* 3:1021–1032
- Lal K, Bromley E, Oaks R, Prietro JH, Sanderson SJ, Kurian D, Hunt L, Yates JR 3rd, Wastling JM, Sinden RE, Tomley FM (2009) Proteomic comparison of four *Eimeria tenella* life-cycle stages: unsporulated oocyst, sporulated oocysts, sporozoite and second-generation merozoite. *Proteomics* 9:4566–4576
- Li L, Brunick BP, Kissinger JC, Pape D, Tang K, Cole RH, Martin J, Wylie T, Dante M, Fogarty L et al (2003) Gene discovery in

- Apicomplexa as revealed by EST sequencing and assembly of comparative gene data base. *Genome Res* 13:443–454
- Marugan-Hernandez V, Alvarez-Garcia G, Tomley F, Hemphill A, Regidor-Cerrillo J, Ortega-Morre LM (2011) Identification of novel rhoptry proteins in *Neospora caninum* by LC/MS analysis of subcellular fractions. *J Proteome* 74:629–642
- Miska KB, Fetterer RH, Barfield RB (2004) Analysis of transcripts expressed by *Eimeria tenella* oocysts using subtractive hybridization. *J Parasitol* 90:1245–1252
- Miska KB, Fetterer RH, Rosenberg GH (2008) Analysis of transcripts from intracellular stages of *Eimeria acervulina* using expressed sequenced tags. *J Parasitol* 94:462–466
- Ng ST, Jangi MS, Shirley MW, Tomley FM, Wan KL (2002) Comparative EST analysis provides insights into gene expression in two asexual developmental stages of *Eimeria tenella*. *Exp Parasitol* 101:168–173
- Oakes RD, Kurian D, Bromley E, Ward C, Lal K, Blake DP, Reid AJ, Pain A, Sinden RE, Wastling JM, Tomley FM (2013) The rhoptry proteome of *Eimeria tenella* sporozoites. *Int J Parasitol* 43:181–188
- Rodger MN, Dubremetz JF, Delplace P, Tonchin G, Vernes A (1988) Characterization of a 225 kilodalton protein of *Plasmodium falciparum*. *Mol Biochem Parasitol* 27:135–141
- Ryan R, Shirley M, Tomley F (2000) Mapping and expression of microneme genes in *Eimeria tenella*. *Int J Parasitol* 30:1493–1499
- Sasai K, Fetterer RH, Lillhoj H, Matusra S, Constantinoiu CC, Matsubayashi M, Tani H, Baba E (2008) Characterization of monoclonal antibodies that recognize the *Eimeria tenella* microneme protein MIC2. *J Parasitol* 94:1432–1434
- Schwarz RS, Evans JD (2013) Single and mixed-species trypanosome and microsporidia infections elicit distinct, ephemeral cellular and humoral immune responses in honey bees. *Develop Comp Immunol* 40:300–310
- Schwarz RS, Fetterer RH, Rosenberg GH, Miska KB (2010) Coccidian merozoite transcriptome analysis from *Eimeria maxima* in comparison to *Eimeria tenella* and *Eimeria acervulina*. *J Parasitol* 96:49–57
- Tomley FM, Soldati DS (2001) Mix and match modules: structure and function of microneme proteins in Apicomplexan parasites. *Trends Parasitol* 17:81–88
- Wan KL, Chong SP, Ng T, Shirley MW, Tomley FM, Jangi MS (1999) A survey of genes in *Eimeria tenella* merozoites by EST sequencing. *Int J Parasitol* 29:1885–1892