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ORIGINAL PAPER

Dynamic development of parasitophorous vacuole of *Eimeria tenella* transfected with the yellow fluorescent protein gene fused to different signal sequences from apicomplexan parasites

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Abstract Intracellular stages of *Eimeria tenella* reside within a membrane-bound parasitophorous vacuole (PV). PVs of apicomplexan parasites like *E. tenella* play important roles in nutrient acquisition, multiplication, and evasion of host immune responses. Different signal sequences from apicomplexan parasites were investigated in the transfected *E. tenella* for their functions in targeting yellow fluorescent protein (YFP) to subcompartments and the dynamic development of the PV of *E. tenella* was studied. Two 5′ terminal signal sequences derived from *Toxoplasma gondii* GRA8 protein and *Plasmodium falciparum* repetitive interspersed family protein, respectively, were confirmed to target YFP to the PVs of the transfected *E. tenella*, suggesting that signal sequences are functionally conserved among Apicomplexa. Three structurally different types of PVs were observed during the endogenous development of the transfected *E. tenella* in vitro. In addition, three subcompartments in the PV, namely, membranous extensions into the host cell cytosol, membranous extensions into the vacuolar lumen, and particle-like bodies, were detected during schizogony of the parasite.

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Introduction

Intracellular stages of apicomplexan parasites reside within a membrane-bound parasitophorous vacuole (PV). The membrane of PVs derives from the plasma membrane of host cells and becomes non-fusigenic for lysosomes or any other vacuoles or vesicles as a result of elimination of host cell proteins and incorporation of parasite proteins (Beyer et al. 2002). Specialized secretory organelles, rhoptries, and dense granules of apicomplexan parasites are believed to be involved in the formation and maintenance of PVs soon after microneme-mediated entry into host cells (Carruthers and Sibley 1997; Baum et al. 2006). The maturation pattern of the PV is parasite-specific and differs within the same genera and species and even at different stages of endogenous development (Beyer et al. 2002). Among Apicomplexa, *Plasmodium*, *Toxoplasma*, and *Eimeria* represent three different types of PVs in terms of structure, transformation, and host cell specificity. Up to now, PVs of different parasite genera and species have been examined to a different extent. Most researches have been directed to the biogenesis, formation, and function of PV harboring *Toxoplasma* or *Plasmodium* because these parasites are important pathogens in humans as well as animals (Cesbron-Delauw et al. 2008; Ravindran and Boothroyd 2008; Saliba and Kirk 2001). However, rather little is known about the PV of *Eimeria* species.

In this study, we first investigated the dynamic development of PVs harboring transfected *Eimeria tenella* expressing a chimeric protein comprising an N-terminal signal peptide of the *Toxoplasma gondii* GRA8 protein appended to the yellow fluorescent protein (YFP). We showed cross-species conservation of *T. gondii* GRA8 signal peptide in

the function of targeting this protein into the PV of *E. tenella*. We further illustrated this conserved protein-targeting machinery among Apicomplexa using another 5′ terminal signal sequence from *Plasmodium falciparum* repetitive interspersed family (RIFIN) protein.

Materials and methods

Plasmid constructs

Three plasmids, namely, pH$_{-90}$-2E-A3′, pHgra8-2E-A3′, and pHrifin-2E-A3′, were constructed by polymerase chain reaction (PCR) using the plasmid pH-2E-A3′ (Fig. 1, B2) as the template, which was previously constructed in our laboratory and contains a tandem repeat *yfp* as the reporter gene flanked by a histone 4 promoter incorporating a 90-bp nucleus location signal sequence (NLS) and an actin 3′ untranslated region (Hao et al. 2007). Plasmid pH$_{-90}$-2E-A3′ (Fig. 1, A2) was constructed by removing the 90-bp NLS from the promoter of the pH-2E-A3′ plasmid as follows. The histone 4 promoter without NLS was amplified by PCR from the pH-2E-A3′ plasmid with the primers 5′-CAGAGATCTAACCAGCAAAGGTAGCAAC-3′ and 5′-CTAGGTACCCATTTTGGTTTTCTATGGAAC-3′. The resulting fragment, bearing the *Bgl*II and *Kpn*I restriction sites, was cloned into the pEASY-Blunt Simple Cloning Vector (TransGen Biotech, Beijing, China) and

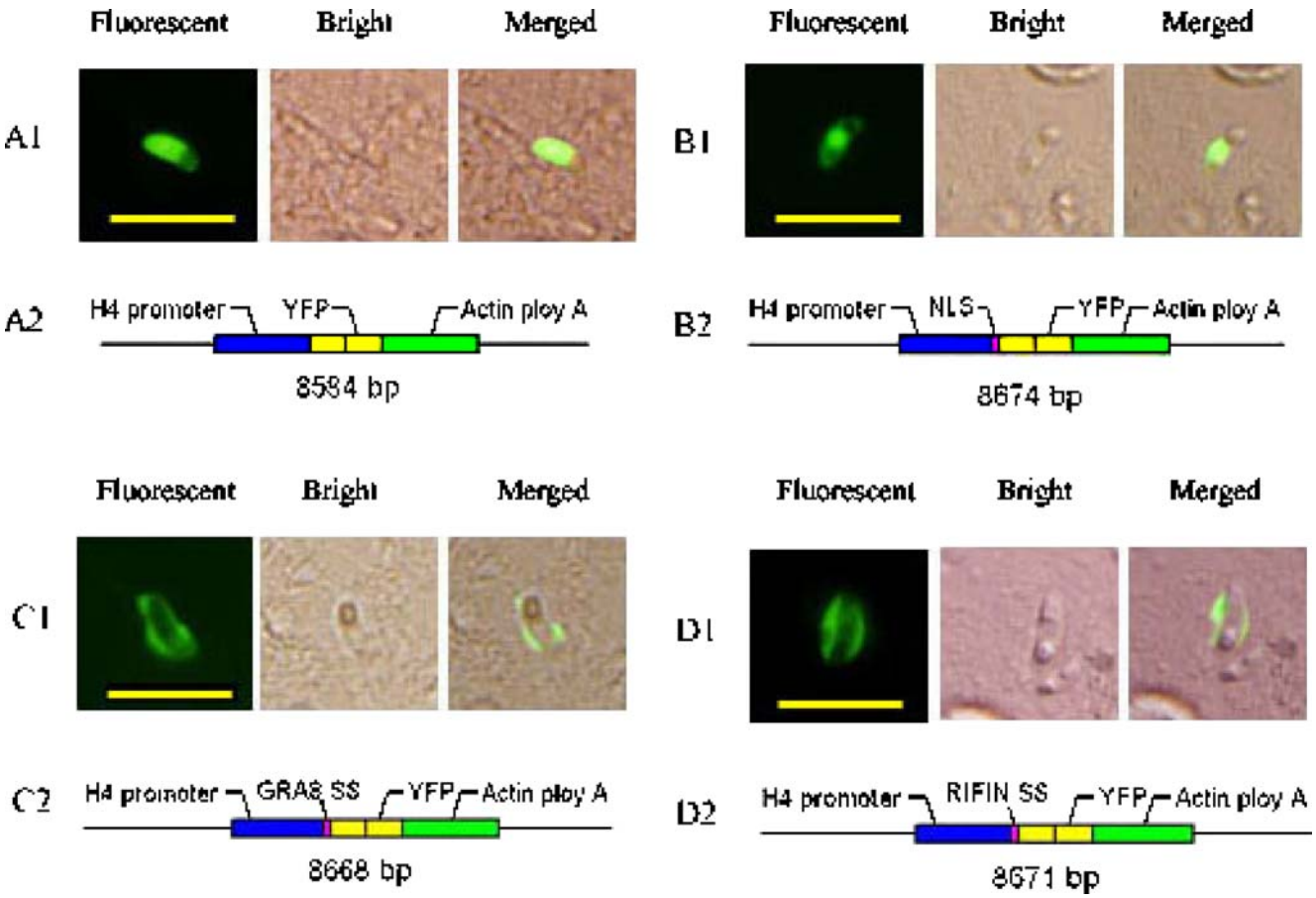

Fig. 1 Different 5′ terminal signal sequences appending the histone 4 promoter-targeted YFP to different subcompartments of the transfected *E. tenella*. *A1* Without additional signal sequence, YFP was expressed into cytosol of transfected *E. tenella* sporozoite in PCKCs. *A2* Schematic construct of pH$_{-90}$-2E-A3′ showing histone 4 (*H4*) promoter from *E. tenella*, tandem *yfp* genes, and an actin poly A tail from *E. tenella*. *B1* NLS of *E. tenella H4* gene-targeted YFP into the nucleus of the transfected *E. tenella* sporozoite in PCKCs. *B2* Schematic construct of plasmid pH-2E-A3′ showing the H4 promoter from *E. tenella* appended by an NLS of *E. tenella*, tandem *yfp* genes, and an actin poly A tail from *E. tenella* (*Actin ploy A*). *C1* 5′ terminal signal sequence of *T. gondii GRA8* gene-targeted YFP into PV of the transfected *E. tenella* sporozoite in PCKCs. *C2* Schematic construct of pHgra8-2E-A3′ showing H4 promoter from *E. tenella* appended by a 5′ terminal signal sequence of *T. gondii GRA8* gene (*GRA8 SS*), tandem *yfp* genes, and an actin poly A tail from *E. tenella*. *D1* 5′ terminal signal sequence of the *P. falciparum* RIFIN protein gene-targeted YFP into the PV of transfected *E. tenella* sporozoite in PCKCs. *D2* Schematic construct of pHrifin-2E-A3′ showing H4 promoter from *E. tenella* appended by a 5′ terminal signal sequence of *P. falciparum RIFIN* gene (*RIFIN SS*), tandem *yfp* genes (YFP), and an actin poly A tail from *E. tenella*. *Fluorescent* fluorescent field image, *Bright* bright field image, *Merged* merged image. *Bar*=20 μm

then placed upstream of the *yfp* gene in the pH-2E-A3′ plasmid.

Plasmids, pHgra8-2E-A3′ (Fig. 1, C2) and pHrifin-2E-A3′ (Fig. 1, D2), were constructed, respectively, by replacing the NLS with the 84- or 153-bp 5′ terminal portion (including the signal sequence) of the *GRA8* gene of *T. gondii* or the *RIFIN* gene of *P. falciparum*. The signal sequence of the *T. gondii GRA8* gene was fused to the histone 4 promoter without NLS by PCR with an upper primer (5′-CAGAGATCTAACCAGCAAAGGTAGCAAC-3′) and two overlapping lower primers (5′-GCGAAGAC CACGAACACCGTGGCCGAAACACGCAATGG TAAAGCCATTTTGGTTTTCTATGGAACAG-3′ and 5′-CTAGGTACCCAAAGGACCGTTCATGGCGCGAGC TACACCAAAGACAGCGAAGACCACGAA CACCGTGGC-3′). The signal sequence of the *RIFIN* gene was fused to histone 4 promoter without NLS by PCR with an upper primer (5′-CAGAGATCTAACCAGCAAAGG-TAGCAAC-3′) and two overlapping lower primers (5′-TATATAATGGAAAGAAAAATAATAATATTT TAGTG TAGTGCAGTTTCATTTTGGTTTTCTATGGAAC-3′ and 5′-CTAGGTACCGTCACATTCACATAATGATC TATTGGTTTGCACCAATATATATAATGGAAA GAAAAATA-3′). The resulting fragments, bearing the *Bgl*II and *Kpn*I restriction sites, were cloned into the pEASY-Blunt simple cloning vector and then placed upstream of the *yfp* gene in the pH-2E-A3′ plasmid. All the inserts above were sequenced in both directions using the fluorescent dideoxynucleotide termination method. Plasmids, pH-2E-A3′ and pH_{-90}-2E-A3′, were used as controls in the study.

Parasites

Oocysts of *E. tenella* BJ strain were maintained, isolated, and sporulated according to the method of Long (1982). Sporozoites were prepared from purified oocysts by grinding and then excystation from sporocytes using a trypsin–bile solution (10% (*v*/*v*) chicken bile and 0.75% (*m*/*v*) trypsin in PBS, pH 7.4) (Schmatz et al. 1984). The released sporozoites were purified by DE-52 anion exchange chromatography and then resuspended in a complete cytomix buffer (10 mM K_2HPO_4:KH_2PO_4, pH 7.6; 120 mM KCl; 0.15 mM $CaCl_2$; 25 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; 2 mM ethylene glycol tetraacetic acid; 5 mM $MgCl_2$; 2 mM adenosine triphosphate; and 5 mM glutathione).

Transfection

The restriction enzyme-mediated integration (REMI) method was used to transfect *E. tenella* sporozoites with the constructed plasmids (Liu et al. 2008). Briefly, a 800-μL complete cytomix mixture consisting of 1×10^7 freshly purified sporozoites, 50 μg plasmid pH_{-90}-2E-A3′, pHgra8-2E-A3′, pHrifin-2E-A3′, or pH-2E-A3′ linearized by the restriction enzyme *Bgl*II (100 IU) was electroporated using a Gene Pulser Xcell™ Electroporation System (BioRad, Hercules, USA) at 2.0 kV and 25 μF. The electroporated sporozoites were allowed to stand for 20 min at room temperature before cultivation in primary chicken kidney cells (PCKCs).

Cultivation and investigation of transfectants

The PCKCs were prepared from 2-week-old chickens 3 days before transfection according to the method of Taylor and Baker (1978) with minor modification. The PCKCs were firstly grown in RPMI1640 (Gibco, Grand Island, USA) supplemented with 10% (*v*/*v*) fetal bovine serum (FBS), 200 U mL^{-1} penicillin, and 20 mg mL^{-1} streptomycin for 24 h at 41°C in an atmosphere containing 5% CO_2. Afterwards, the cells were cultured in the same medium but with 5% FBS for 2 days before incubation with the transfected sporozoites.

The transfected *E. tenella* was investigated using a fluorescence microscope (Olympus, Tokyo, Japan) with 488-nm excitation and 508-nm emission filters, under which the expressed fluorescence appeared green (Hao et al. 2007).

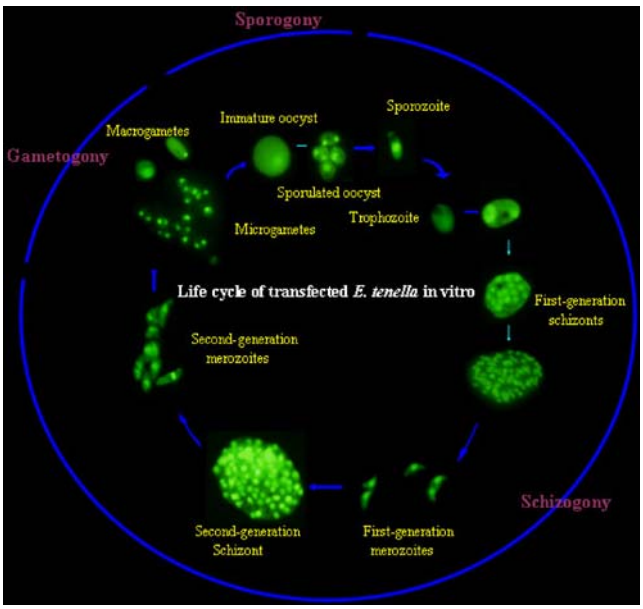

Fig. 2 Under the promoting *E. tenella* histone 4 promoter, YFP was constitutively expressed throughout the life cycle of the transfected *E. tenella* in vitro. Plasmid pH-2E-A3′ was used in the transfection of *E. tenella* (Shi et al. 2008)

Results and discussion

The histone 4 promoter of *E. tenella* was demonstrated as a powerful constitutively expressing promoter, which facilitated the expression of YFP throughout the life cycle of the transfected *E. tenella* (Fig. 2) (Shi et al. 2008). In the current study, we firstly showed that 5′ terminal signal sequences played essential roles in sorting proteins to different subcompartments of the transfected *E. tenella* with the histone 4 promoter. In *E. tenella* transfected with plasmid pH$_{-90}$-2E-A3′ without any additional signal sequence, the expressed YFP was confined in the cytosol (Fig. 1, A1). In parasites transfected with plasmid pH-2E-A3′ containing a NLS between the histone 4 promoter and *yfp*, YFP was targeted into the nucleus of *E. tenella* (Fig. 1, B1). However, when the 5′ terminal signal sequences from *T. gondii* GRA8 or *P. falciparum* RIFIN protein genes were introduced replacing the NLS as in plasmids pHgra8-2E-A3′ or pHrifin-2E-A3′, YFP was expressed into the PV (Fig. 1, C1 and D1). As secretory proteins, GRA8 and RIFIN proteins were expressed into or beyond PVs of *T. gondii* and *P. falciparum*, respectively (Carey et al. 2000; Marti et al. 2004). Targeting of YFP into PVs of *E. tenella* by the two signal sequences from GRA8 and RIFIN proteins suggests that these sequences are functionally conserved across Apicomplexa parasites.

On the basis of the results above, we further investigated the dynamic development of the PV harboring *E. tenella* transfected with the GRA8 signal sequence and YFP. Three structurally different PVs were observed during the endogenous development of the parasite in vitro. The PV of sporozoites was characterized by a narrow lumen and few

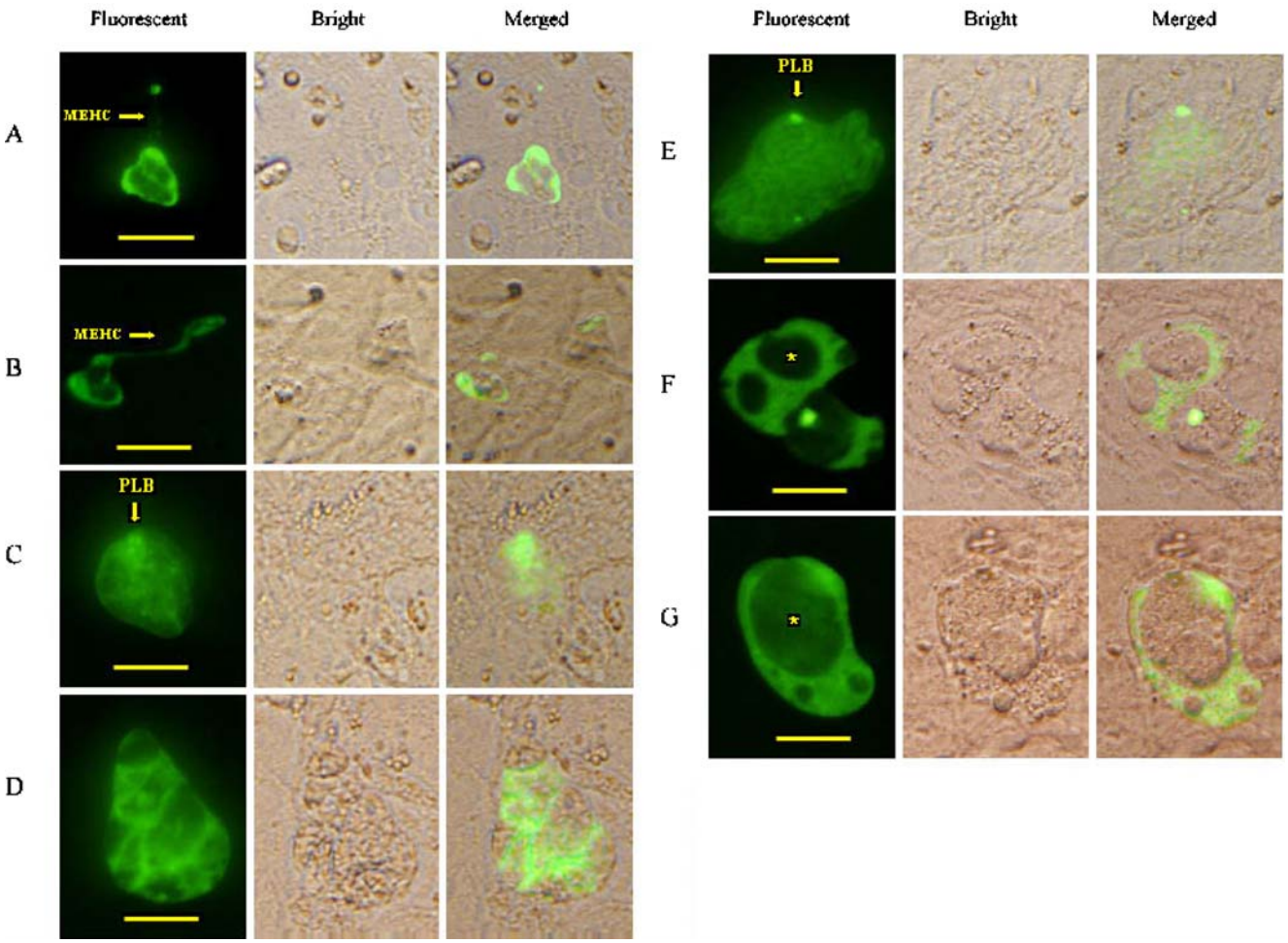

Fig. 3 Dynamic development of the PV of *E. tenella* transfected with plasmid pHgra8-2E-A3′ carrying a 5′ terminal signal sequence of *T. gondii GRA8* gene. **a**, **b** PVs harboring immature first-generation schizonts, showing MEHC. **c** PV of first-generation schizonts, showing PLB and numerous MEVL. **d** PV of a schizont, showing complicated MEVL within the PV. **e** PV of merozoites of a second-generation schizont, showing profuse MEVL around merozoites. **f**, **e** Enlarged and matrix-filling PVs harboring gamonts (*asterisk*). *Fluorescent* fluorescent field image, *Bright* bright field image, *Merged* merged image. *Bar*=20 μm

visible subcompartments (Fig. 1, C1). It was only detected 3 days post-inoculation despite that PV could actually be formed soon after entry of the sporozoite into the host cells within the first few hours after infection. This probably was because of delayed or lower YFP expression into the nascent PV. As the parasite developed into schizogony, three subcompartments were observed in the PVs around schizonts. The first subcompartment was thin membranous extensions of PV into the host cytosol (MEHC) (Fig. 3a,b). Same structures, called duct-like structures, were observed in stages of sporozoites and immature schizonts but not mature schizonts (first and second generation) or gametogony by Zgrzebski et al. (1993), who described that the duct-like structures always extend from the posterior pole of the sporozoite and the PV, marking the pathway of the parasite after host cell invasion. We observed the same phenomena in our study. We postulate that MEHC provides a trafficking pathway between the parasite and the host cell via which macromolecules can passively diffuse into or out of the parasite. Similar structures were also observed in *T. gondii* and *P. falciparum* (Magno et al. 2005; Schatten and Ris 2004; Adisa et al. 2003). The second compartment was membranous extensions into the vacuolar lumen of the PV (MEVL) (Fig. 3d). This was the first time that existence of numerous MEVL in *E. tenella* was observed. The third subcompartment observed in schizogony of the parasite was round, particle-like bodies (PLB) in the lumen of PV (Fig. 3c,e). As the parasite developed into gametogony, the lumen of the PV enlarged, MEVL seemed to disappear, and the MEHC were seldom visible under fluorescence microscopy (Fig. 3f,g). PVs harboring zygotes or oocysts were not detected in our study probably because of very low YFP expression into the degraded PV. In addition, no fluorescent zoites was found outside the PCKCs throughout all the developmental stages of the transfected *E. tenella*, indicating that YFP is only targeted to the PVs. For the parasites within the PCKCs, fluorescence labeling of dense granules was not observed despite that YFP was obviously detected in the PVs of the transfected parasites. In *T. gondii*, any soluble protein (including GFP or YFP) recombinantly fused to a signal peptide is first delivered to dense granules and then targeted to distinct subcompartments of the PVs (Joiner and Roos 2002). It was believed that a default route was involved in delivering soluble proteins into dense granules and subsequently targeting them into the PV for *T. gondii* (Kaasch et al. 2000). The pathway for the GRA8 signal peptide fused YFP targeting to PVs in transfected *E. tenella* is to be determined in a future study.

Our study firstly provided an insight into the dynamic development of the PV harboring *E. tenella* and proved that N-terminal signal sequences from secretory proteins of apicomplexan parasites were determinant in protein trafficking to the PV. In addition, our results suggested that these signal sequences are conserved across species of Apicomplexa in the post-secretory targeting of proteins to the PV. Our findings are valuable in studying protein trafficking in apicomplexan parasites and developing *E. tenella* as a convenient vaccine vector delivering viral and/or bacterial antigens.

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References

Adisa A, Rug M, Klonis N, Foley M, Cowman AF, Tilley L (2003) The signal sequence of exported protein-1 directs the green fluorescent protein to the parasitophorous vacuole of transfected malaria parasites. J Biol Chem 278(8):6532–6542

Baum J, Papenfuss AT, Baum B, Speed TP, Cowman AF (2006) Regulation of apicomplexan actin-based motility. Nat Rev Microbiol 4(8):621–628

Beyer TV, Svezhova NV, Radchenko AI, Sidorenko NV (2002) Parasitophorous vacuole: morphofunctional diversity in different coccidian genera (a short insight into the problem). Cell Biol Int 26(10):861–871

Carey KL, Donahue CG, Ward GE (2000) Identification and molecular characterization of GRA8, a novel, proline-rich, dense granule protein of *Toxoplasma gondii*. Mol Biochem Parasitol 105(1):25–37

Carruthers VB, Sibley LD (1997) Sequential protein secretion from three distinct organelles of *Toxoplasma gondii* accompanies invasion of human fibroblasts. Eur J Cell Biol 73:114–1123

Cesbron-Delauw MF, Gendrin C, Travier L, Ruffiot P, Mercier C (2008) Apicomplexa in mammalian cells: trafficking to the parasitophorous vacuole. Traffic 9(5):657–664

Hao L, Liu X, Zhou X, Li J, Suo X (2007) Transient transfection of *Eimeria tenella* using yellow or red fluorescent protein as a marker. Mol Biochem Parasitol 153(2):213–215

Joiner KA, Roos DS (2002) Secretory traffic in the eukaryotic parasite *Toxoplasma gondii*: less is more. J Cell Biol 157(4):557–563

Kaasch AJ, Joiner KA (2000) Protein-targeting determinants in the secretory pathway of apicomplexan parasites. Curr Opin Microbiol 3(4):422–428

Liu X, Shi T, Ren H, Su H, Yan W, Suo X (2008) Restriction enzyme-mediated transfection improved transfection efficiency in vitro in apicomplexan parasite *Eimeria tenella*. Mol Biochem Parasitol 161(1):72–75

Long PL (1982) The biology of the coccidian. Part 4. Ultrastructure. University Park Press, Baltimore, pp 117–155

Magno RC, Straker LC, de Souza W, Attias M (2005) Interrelations between the parasitophorous vacuole of *Toxoplasma gondii* and host cell organelles. Microsc Microanal 11(2):166–174

Marti M, Good RT, Rug M, Knuepfer E, Cowman AF (2004) Targeting malaria virulence and remodeling proteins to the host erythrocyte. Science 306(5703):1930–1933

Ravindran S, Boothroyd JC (2008) Secretion of proteins into host cells by apicomplexan parasites. Traffic 9(5):647–656

Saliba KJ, Kirk K (2001) Nutrient acquisition by intracellular apicomplexan parasites: staying in for dinner. Int J Parasitol 31(12):1321–1330

Schatten H, Ris H (2004) Three-dimensional imaging of *Toxoplasma gondii*—host cell interactions within the parasitophorous vacuole. Microsc Microanal 10(5):580–585

Schmatz DM, Crane MS, Murray PK (1984) Purification of *Eimeria* sporozoites by DE-52 anion exchange chromatography. J Protozool 31:181–183

Shi T, Liu X, Hao L, Li J, Abdel G, Abdille M, Suo X (2008) Transfected *Eimeria tenella* could complete its endogenous development in vitro. J Parasitol 11:1

Taylor AER, Baker JR (1978) Methods of cultivating parasites in vitro. Academic, London, pp 118–121

Zgrzebski G, Raether W, Hofmann J, Entzeroth R (1993) Secretion of an *Eimeria tenella* sporozoite antigen during host-cell invasion: visualization of the parasitophorous vacuole membrane and parasitophorous duct-like structures. Parasitol Res 79 (1):77–79