Parasitol Res (2013) 112:2105–2109
DOI 10.1007/s00436-013-3307-5

SHORT COMMUNICATION

Expression of *Toxoplasma gondii* dense granule protein7 (GRA7) in *Eimeria tenella*

Guangwen Yin · Mei Qin · Xianyong Liu · Jingxia Suo · Xun Suo

Received: 19 August 2012 / Accepted: 18 January 2013 / Published online: 31 January 2013

Abstract Dense granules are specialized secretory organelles of Apicomplexa parasites; the dense granule (GRA) proteins are believed to play a role in intracellular survival and the nutrient/waste exchange mechanism with the host cell. Until now, limited information is available concerning the characterization of GRA proteins in *Eimeria*. *Eimeria tenella* and *Toxoplasma gondii* are apicomplexan protozoa and share many similarities in biology and genomics. We hypothesized that GRA proteins from *T. gondii* could be expressed and have a similar function in *E. tenella*. To confirm the expression and localization of the GRA protein in *T. gondii* and *E. tenella*, a transient transfection strategy was used to express *T. gondii* GRA7 tagged with yellow fluorescent protein (YFP) (GRA7-YFP); *T. gondii* tachyzoites were transfected with the plasmid pTgtubGRA7-YFP/sagCAT, and *E. tenella* sporozoites were transfected with the pEtmic1GRA7-YFP/act construct. The results show that fluorescence can be expressed mainly into the parasitophorous vacuoles (PVs) of the *T. gondii*. GRA7 of *T. gondii* can also be expressed in *E. tenella* and can lead the fluorescence protein into the PVs of the parasites and the cavity of the sporocysts. As for the extracellular stage, YFP gathered to form small particles in the released merozoites and sporozoites, suggesting a localization of the secretory organelles of *E. tenella*. These results suggest that GRA proteins have a conserved function across species of Apicomplexa in targeting proteins to the PVs.

G. Yin · M. Qin · X. Liu · J. Suo · X. Suo
National Animal Protozoa Laboratory & College of Veterinary Medicine, China Agricultural University, Beijing 100193, China

X. Liu · X. Suo (✉)
Key Laboratory of Zoonosis of Ministry of Agriculture & College of Veterinary Medicine, China Agricultural University, Beijing 100193, China
e-mail: suoxun@cau.edu.cn

Introduction

Dense granules (DGs) are characteristic spherical organelles present in the merozoites of *Plasrnodium* spp., *Sarcocystis* spp., and *Toxoplasma gondii*. (Daszak et al. 1993). DGs have mostly been studied in the genus *Sarcocystis*, from which they have been isolated by subcellular fractionation and characterized and in which they were subsequently shown to be released into the secondary parasitophorous vacuole after host cell invasion (Leriche and Dubremetz 1990). DGs surrounded by a unique membrane are specialized secretory organelles of Apicomplexa parasites. Their number varies from a few DGs (*Eimeria*) to approximately 20 (*Toxoplasma*, *Neospora*) (Mercier et al. 2005). After invasion of the host cell, the contents of DGs are secreted into the parasitophorous vacuole (PVs); the dense granule proteins are considered to remodel the PVs and have important functions in parasite–host interrelation (Cesbron-Delauw et al. 1996).

Among the GRA proteins, GRA7 is expressed by all infectious forms of *Toxoplasma* (Ferguson et al. 1999), and anti-GRA7 antibodies are generated during murine and human infection (Carey et al. 2000; Neudeck et al. 2002). GRA7 has been detected on the surface of infected host cells (Neudeck et al. 2002). Within the infected host cell, GRA7 localizes to the PV lumen, the PV membrane, and strands extending from the PV membrane into the host cytosol (Fischer et al. 1998; Coppens et al. 2006).

Until now, limited information is available concerning the characterization of GRA proteins in *Eimeria* (Bonnin et al. 1995), with no report of sequence or function for these proteins. Therefore, we want to know if GRA7 of *T. gondii* could be expressed in *E. tenella*. In this paper, we report the expression of the GRA7 in *E. tenella* and localization of its protein products to different compartments of different stages of the parasites.

Materials and methods

Parasite and cell culture

E. tenella (BJ strain) was maintained and propagated in coccidia-free, 2–5-week-old AA broilers (Yan et al. 2009; Yin et al. 2011). Oocysts were collected from feces of chickens 6–9 days post-infection and were isolated, purified, and sporulated as described previously (Long et al. 1976; Huang et al. 2011). Sporozoite excystation and purification were achieved using a previously described method. Freshly purified *E. tenella* sporozoites were resuspended in cytomix buffer supplemented with 2 mM ATP and 5 mM glutathione (van den Hoff et al. 1992; Kelleher and Tomley 1998).

Primary chicken kidney (PCK) cells were used for in vitro transient transfection experiments. PCKs were cultured in DMEM medium supplemented with fetal bovine serum (10 %, *v*/*v*) and 1,000 U penicillin/streptomycin in a humidified atmosphere of 5 % CO_2 at 41 °C.

Plasmid constructs

The vector pTgtubYFP-YFP/sagCAT plasmid (Gubbels et al. 2003) carries the tandem yellow fluorescent protein (YFP) gene and possesses the upstream region of the *T. gondii* tubulin gene. The coding sequence of GRA7 protein was amplified by PCR from the cDNA of the *T. gondii* (RH strain) parasites with the primers 5′-AGATCTATGGCCC-GACACGCAATTT-3′ and 5′-CCTAGGCTGGCGGG-CATCC TCCCCA-3′. The resulting fragment, bearing the *BglII* and *AvrII* restriction sites, was cloned into the pEASY-Blunt Simple Cloning Vector (TransGen Biotech, Beijing, China) and then inserted to the N-terminal of the YFP gene of the ptubYFP-YFP/sagCAT plasmid. The resulting recombinant plasmid was named pTgtubGRA7-YFP/sagCAT (Fig. 1). The pEtmic1GRA7-YFP/act plasmid was constructed from the pEtmic1YFP-YFP/act plasmid (Zou et al., unpublished data). First, GRA7-YFP fusion gene was amplified by PCR from the plasmid pTgtubGRA7-YFP/sagCAT with the primers 5′-GATGGTACCATGGCCCGACACG CAATTT-3′ and 5′-GGGCGGCCGCCTAAAGCTTCTTGTACAGCT-3′. The product was cloned into *KpnI* and *NotI*-digested pEtmic1YFP-YFP/act to produce the final expression vector pEtmic1GRA7-YFP/act (Fig. 1).

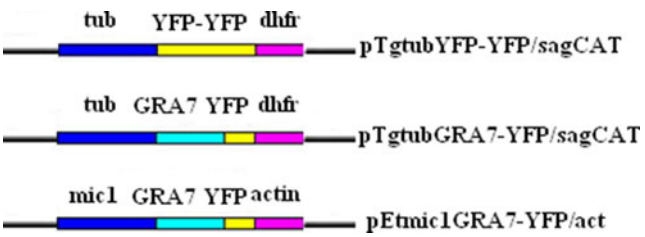

Fig. 1 Schematic diagram of the expression vectors. Plasmid-containing tubulin gene promoter (Tgtub) from *T. gondii* and microneme protein 1 gene promoter (Etmic1) from *Eimeria tenella*, respectively

Transfection

For plasmid transfection, five million sporozoites or tachyzoites, 10 μl linearized plasmid DNA together with 5 μl *SnaBI* were subjected to Nucleofector transfection. After AMAXA (Switzerland) nucleofection (ProgramU-033) (Clark et al. 2008; Yin et al. 2011), the transfected tachyzoites were then inoculated onto Vero (African green monkey kidney) cells in a humidified atmosphere of 5 % CO_2 at 37 °C, and the electroporated sporozoites were inoculated onto 25-cm^2 flasks (Corning, Costar, USA) with PCKCs, which were cultured in DMEM medium supplemented with fetal bovine serum (10 %, *v*/*v*) and 1,000 U penicillin/streptomycin in a humidified atmosphere of 5 % CO_2 at 41 °C.

For the in vivo experiment, 2×10^6 electroporated sporozoites were inoculated via the cloacal route (Clark et al. 2008). The caecal mucous membranes of infected chickens were scraped and viewed under the fluorescence microscope to detect various fluorescent endogenous stages of *E. tenella* at 120 h post-inoculation (p.i.). Oocysts in feces excreted between days 6 and 9 post-inoculation were collected and checked by a fluorescence microscopy (Shi et al. 2008; Yan et al. 2009; Yin et al. 2011).

Fluorescence observation

The transfected *T. gondii* and *E. tenella* were investigated using a fluorescence microscope (Olympus, IX71, Tokyo, Japan) with 488-nm excitation and 508-nm emission filters, under which, the expressed fluorescence appeared green (Hao et al. 2007; Yin et al. 2011).

Results and discussion

Expression of GRA7 in transfected *T. gondii* tachyzoites

To confirm the expression and localization of the GRA7-EYFP fusion protein in *T. gondii* tachyzoites, *T. gondii* tachyzoites were transfected with the pTgtubGRA7-YFP/sagCAT construct. Fluorescence was detected in the transfected parasites throughout the observation period (12–120 h after electroporation) (Fig. 2). At 12 h p.i., only a few tachyzoites displayed weak fluorescence. By 24 h, each tachyzoite expressing YFP multiplied to two or four tachyzoites, all of which remained fluorescent (Fig. 2a). Between 48 and 72 h, greater fluorescence was displayed by the tachyzoites expressing YFP (Fig. 2b, c). Fluorescence was expressed mainly into PVs of the parasites (Fig. 2); this is consistent with the previous report (Ferguson et al. 1999; Carey et al. 2000;

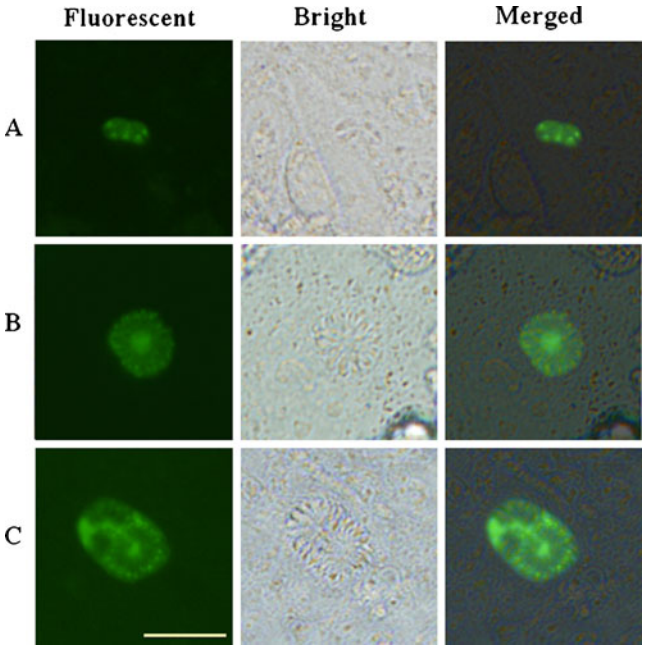

Fig. 2 Expression of GRA7-YFP protein in *T. gondii* transfected with the plasmid pTgtubGRA7-YFP/sagCAT. Transfected tachyzoites were inoculated onto PCK cells and observed by fluorescence microscopy during different times. The images show that the fluorescent protein was targeted into the PV by GRA7. Multiplication of tachyzoites in the vacuoles could be observed post-inoculation. **a** Four tachyzoites at 24 h post-inoculation (p.i.), **b** 16 tachyzoites 48 h p.i., **c** 32 tachyzoites 64 h p.i. Bar=20 μm

Neudeck et al. 2002). When the tachyzoites were transfected with the ptubYFP-YFP/sagCAT plasmid, the fluorescence was uniform, expressed mainly into the cytoplasm of the parasites (Zou et al. 2009).

Transient expression of GRA7 in *E. tenella*

The tubulin gene promoter of *T. gondii* is a strong promoter in *T. gondii*, and it also worked in *E. tenella* (Zou et al. 2009), but the transfection efficiency was so low that it was difficult to get the transgenic parasites, especially the oocysts. Then, we used the MIC1 promoter of *E. tenella* to drvie the expression of the foreign gene. At 16 h p.i., sporozoites expressing YFP were observed in parasites transfected with pEtmic1GRA7-YFP/act. Fluorescence was expressed mainly into PVs of the parasites (Fig. 3a), immature first-generation schizonts expressing YFP were observed at 42–60 h p.i. (Fig. 3b), and thin membranous extensions of PV into the host cytosol (MEHC) can also be observed (data not shown). Same structures, called duct-like structures, were observed in stages of sporozoites and immature schizonts 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. In a previous study by Shi et al. (2009), they also observed MEHC, and they postulated that MEHC provides a trafficking

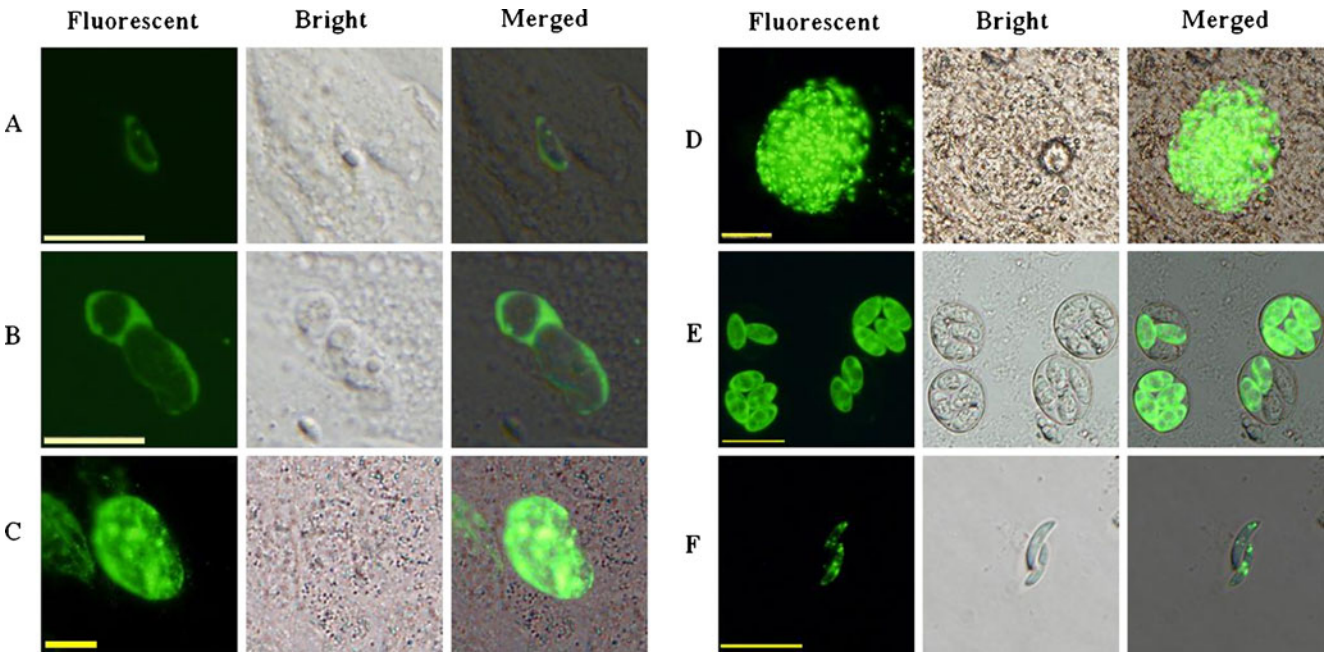

Fig. 3 Expression of GRA7-YFP protein in *E. tenella* transfected with the plasmid pEtmic1GRA7-YFP/act. Transfected sporozoites were inoculated with host cells (PCK cells) and observed by fluorescence microscopy during different times. **a** PV harboring sporozoite. **b** Immature first-generation schizonts in PVs. **c** Immature second-generation schizonts in PVs. **d** A second-generation schizont with releasing merozoites. **e** Sporulated oocysts with the fluorescent protein into the cavity of the sporocysts. **f** Released sporozoites from the oocysts. Bar=20 μm

pathway between the parasite and the host cell via which macromolecules can passively diffuse into or out of the parasite (Shi et al. 2009). The immature second-generation schizonts still expressed the YFP into the PVs (Fig. 3c), but the mature schizonts expressed YFP into the parasite to form small particles (Fig. 3d). In the stage of the sporulated oocysts, YFP was targeted into the cavity of the sporocysts (Fig. 3e). As for the extracellular sporozoites, YFP gathered to form small particles in the parasite (Fig. 3f). When the sporozoites were transfected with the pEtmic1YFP-YFP/act plasmid, which has no GRA7 gene sequence, the fluorescence was uniform in the transfected parasites, suggesting a cytoplasmic localization of the reporter protein (data not shown).

In a previous study, fluorescent protein can be targeted into the PVs of *E. tenella* by signal peptide of GRA8 derived from *T. gondii*, but no fluorescent zoites and oocysts were observed, indicating that YFP is only targeted to the PVs in that system (Shi et al. 2009).

In our study, we utilized the GRA7 coding sequence, including the signal sequence to target the fluorescent protein, and we got the transgenic oocysts. In the intracellular stage, GRA7-YFP fusion protein was targeted into the PVs of the sporozoites and the immature schizonts; during the extracellular stage, YFP gathered to form small particles in the released merozoites and sporozoites; the small particles in the parasites may be the secretory organelles of the *E. tenella*, but the detailed pathway needs to be determined in future study. Interestingly, the fusion protein could be secreted into the cavity of the sporocysts in sporulated oocysts. The ability to secret foreign proteins by GRA7 is of great importance for developing transgenic eimerian parasites into a eukaryotic vaccine vector, as researches indicated that secreted antigens were superior in inducing protective immunity (Hess et al. 1996; Shen et al. 1998). The experiments described in this paper suggest that a system using *E. tenella* to study the localization of *T. gondii* antigen is appropriate; in addition, our results suggested that GRA proteins have a conserved function across species of Apicomplexa in targeting proteins to the PV.

Acknowledgments This study was supported by the National Natural Science Foundation of China (30871862), the National High Technology Research and Development Program of China (2011AA10A209) ,Yangtze River Scholar and Innovation Research Team Development Program (IRT0945), and Research and Innovation Project for College Graduates of China Agricultural University (KYCX2011052).All experimentations using chickens were carried out in compliance with the current Chinese legislation, and due attention was paid to the welfare of experimental chickens..

References

Bonnin A, Gut J, Dubremetz JF, Nelson RG, Camerlynck P (1995) Monoclonal antibodies identify a subset of dense granules in *Cryptosporidium parvum* zoites and gamonts. J Eukaryot Microbiol 42(4):395–401

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

Cesbron-Delauw MF, Lecordier L, Mercier C (1996) Role of secretory dense granule organelles in the pathogenesis of toxoplasmosis. Curr Top Microbiol 219:59–65

Clark JD, Billington K, Bumstead JM, Oakes RD, Soon PE, Sopp P, Tomley FM, Blake DP (2008) A toolbox facilitating stable transfection of *Eimeria* species. Mol Biochem Parasitol 162(1):77–86

Coppens I, Dunn JD, Romano JD, Pypaert M, Zhang H, Boothroyd JC, Joiner KA (2006) *Toxoplasma gondii* sequesters lysosomes from mammalian hosts in the vacuolar space. Cell 125(2):261–274

Daszak P, Ball SJ, Pittilo RM, Norton CC (1993) Ultrastructural evidence for dense granule exocytosis by first-generation merozoites of *Eimeria tenella* in vivo. Parasitol Res 79(3):256–258

Ferguson DJ, Jacobs D, Saman E, Dubremetz JF, Wright SE (1999) In vivo expression and distribution of dense granule protein 7 (GRA7) in the exoenteric (tachyzoite, bradyzoite) and enteric (coccidian) forms of *Toxoplasma gondii*. Parasitology 119(Pt 3):259–265

Fischer HG, Stachelhaus S, Sahm M, Meyer HE, Reichmann G (1998) GRA7, an excretory 29 kDa *Toxoplasma gondii* dense granule antigen released by infected host cells. Mol Biochem Parasitol 91 (2):251–262

Gubbels MJ, Li C, Striepen B (2003) High-throughput growth assay for *Toxoplasma gondii* using yellow fluorescent protein. Antimicrob Agents Ch 47:309–316

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

Hess J, Gentschev I, Miko D, Welzel M, Ladel C, Goebel W, Kaufmann SH (1996) Superior efficacy of secreted over somatic antigen display in recombinant *Salmonella* vaccine induced protection against listeriosis. Proc Nat Acad Sci USA 93(4):1458–1463

Huang X, Zou J, Xu H, Ding Y, Yin G, Liu X, Suo X (2011) Transgenic *Eimeria tenella* expressing enhanced yellow fluorescent protein targeted to different cellular compartments stimulated dichotomic immune responses in chickens. J Immunol 187 (7):3595–3602

Kelleher M, Tomley FM (1998) Transient expression of beta-galactosidase in differentiating sporozoites of *Eimeria tenella*. Mol Biochem Parasitol 97(1–2):21–31

Leriche MA, Dubremetz JF (1990) Exocytosis of *Toxoplasma gondii* dense granules into the parasitophorous vacuole after host cell invasion. Parasitol Res 76(7):559–562

Long PL, Millard BJ, Joyner LP, Norton CC (1976) A guide to laboratory techniques used in the study and diagnosis of avian coccidiosis. Folia Vet Lat 6:201–217

Mercier C, Adjogble KD, Daubener W, Delauw MF (2005) Dense granules: are they key organelles to help understand the parasitophorous vacuole of all apicomplexa parasites? Int J Parasitol 35(8):829–849

Neudeck A, Stachelhaus S, Nischik N, Striepen B, Reichmann G, Fischer HG (2002) Expression variance, biochemical and immunological properties of *Toxoplasma gondii* dense granule protein GRA7. Microbes and infection / Institut Pasteur 4:581–590

Shen H, Miller JF, Fan X, Kolwyck D, Ahmed R, Harty JT (1998) Compartmentalization of bacterial antigens: differential effects on priming of CD8 T cells and protective immunity. Cell 92(4):535–545

Shi TY, Liu XY, Hao LL, Li JD, Gh AN, Abdille MH, Suo X (2008) Transfected *Eimeria tenella* could complete its endogenous development in vitro. J Parasitol 94(4):978–980

Shi T, Yan W, Ren H, Liu X, Suo X (2009) Dynamic development of parasitophorous vacuole of *Eimeria tenella* transfected with the yellow fluorescent protein gene fused to different signal sequences from apicomplexan parasites. Parasitol Res 104(2):315–320

van den Hoff MJ, Moorman AF, Lamers WH (1992) Electroporation in "intracellular" buffer increases cell survival. Nuleic Acids Res 20 (11):2902

Yan W, Liu X, Shi T, Hao L, Tomley FM, Suo X (2009) Stable transfection of *Eimeria tenella*: constitutive expression of the YFP-YFP molecule throughout the life cycle. Int J Parasitol 39(1):109–117

Yin G, Liu X, Zou J, Huang X, Suo X (2011) Co-expression of reporter genes in the widespread pathogen *Eimeria tenella* using a double-cassette expression vector strategy. Int J Parasitol 41(8):813–816

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

Zou J, Liu X, Shi T, Huang X, Wang H, Hao L, Yin G, Suo X (2009) Transfection of *Eimeria* and *Toxoplasma* using heterologous regulatory sequences. Int J Parasitol 39(11):1189–1193