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Research Brief

Toxoplasma gondii aspartic protease 1 is not essential in tachyzoites

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ABSTRACT

Aspartic proteases are important virulence factors for pathogens and are recognized as attractive drug targets. Seven aspartic proteases (ASPs) have been identified in *Toxoplasma gondii* genome. Bioinformatics and phylogenetic analyses regroup them into five monophyletic groups. Among them, TgASP1, a coccidian specific aspartic protease related to the food vacuole plasmepsins, is associated with the secretory pathway in non-dividing cells and relocalizes in close proximity to the nascent inner membrane complex (IMC) of daughter cells during replication. Despite a potential role for TgASP1 in IMC formation, the generation of a conventional knockout of the *TgASP1* gene revealed that this protease is not required for *T. gondii* tachyzoite survival or for proper IMC biogenesis.

1. Introduction

The phylum Apicomplexa regroups obligate intracellular protozoan parasites with medical and economic importance such as *Plasmodium falciparum*, the causative agent of malaria and *Toxoplasma gondii*, responsible for toxoplasmosis. *T. gondii* tachyzoites invade almost any nucleated cell and replicate within a non-fusogenic vacuole (Boyle and Radke, 2009). Malaria claims more than one million human lives annually while toxoplasmosis can lead to severe neurological disorders and death in immunocompromised individuals. The unavailability of a vaccine and the spread and intensification of drug resistance have led to a considerable decline in the efficacy of the drugs used to eradicate Apicomplexans. Most available drugs target metabolic pathways but parasite proteases are considered as attractive alternative targets for therapeutic intervention.

Aspartic proteases are common in eukaryotes where they are involved in a wide range of biological functions such as nutrient acquisition and activation of signalling cascades. Aspartic proteases are important virulence factors and considered as potential targets for therapy in *Candida albicans* (Naglik et al., 2003; Hoegl et al., 1999) whereas they are already successfully exploited as targets for therapy against HIV (Wlodawer and Vondrasek, 1998). In Apicomplexans, most data on aspartic proteases concern the causative agent of malaria, *P. falciparum*. Successful use of aspartic protease inhibitors against *Plasmodium* parasites *in vitro* validates *Plasmodium* aspartic proteases as potential drug targets (Bonilla et al., 2007).

A database mining of the apicomplexan genomes allowed the identification of five distinct phylogenetic groups of aspartic proteases (Shea et al., 2007). The genome of *P. falciparum* encodes 10 ASPs termed plasmepsins (PMs), four of which (PfPMI, II, IV and HAP) are involved in haemoglobin degradation within the food vacuole, and hence critically provide amino acids for parasite growth (Bonilla et al., 2007). In addition to haemoglobinase activity, PfPMII might be involved in erythrocyte cytoskeleton remodelling and in egress by cleaving spectrin (Le Bonniec et al., 1999). PMIV, which was previously only demonstrated to function in the food vacuole of asexual stages, was recently localized to the micronemes and at the apical surface of ookinetes. A second role for PMIV is suspected in mosquito midgut invasion and/or development of oocysts from ookinetes (Li et al., 2010). Most recently, PfPMV has been localized to the endoplasmic reticulum (ER) and was demonstrated to be essential for parasite viability and hence represents a new target for therapeutic intervention against malaria. PfPMV cleaves exported proteins at a conserved PEXEL motif allowing translocation of several hundred proteins to the host cell cytoplasm via the ATP driven translocator PTEX to remodel the host cell in order to survive and evade the host response (Boddey et al., 2010; Russo et al., 2010).

In *T. gondii*, among the seven ASPs found in the genome, four are expressed in tachyzoites. TgASP3 and TgASP5 have been localized to the Golgi compartment and TgASP5 is the closest homologue of PfPMV (Shea et al., 2007). In contrast, TgASP1 is a protease only present in *T. gondii* and in *N. caninum* (Fig. S1) suggesting a specific role in these two coccidians. Phylogenetic analysis indicates that TgASP1 clusters with the type II transmembrane PMs that localize

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to the food vacuole and are implicated in haemoglobin degradation, but the phylogenetic tree has weak bootstrap support. However, TgASP1 must fulfil a distinct function since *T. gondii* does not digest haemoglobin and does not possess a food vacuole. Like all plasmepsins previously characterized except PMV, TgASP1 is synthesised as a zymogen, which is processed by autocatalytic activity or by the action of additional proteases (Drew et al., 2008). TgASP1 was shown to localize to a novel punctuate compartment associated with the secretory pathway in non-dividing cells. During replication, TgASP1 relocalizes to the nascent inner membrane complex (IMC) of the daughter cells before coalescing again at the end of the cell division (Shea et al., 2007). A potential role in endodyogeny was postulated by the absence of homologues in others Apicomplexans known not to undergo endodyogeny. Here we describe the successful disruption of the first gene coding for an aspartic protease in *T. gondii*, *TgASP1*.

2. Materials and methods

2.1. Cell culture

T. gondii tachyzoites (RH wild type strain hxgprt-, Donald et al., 1996) were grown in human foreskin fibroblasts (HFF) monolayer cells or in Veros cells (African green monkey kidney cells in Dulbecco's Modified Eagle's Medium DMEM, GIBCO, Invitrogen) supplemented with 10% fetal calf serum (FCS), 2 mM glutamine and 25 μg/ml gentamicin.

2.2. Cloning of DNA construct

Genomic DNA was prepared using the Promega Wizard SV genomic DNA purification system. TgASP1 genomic sequence was obtained from ToxoDB database. PCR has been performed according to the manufacturer's instructions using the Takara LaTaq. The 5′ flanking region of TgASP1 (1.9 kb upstream the start codon) has been amplified (Table S1) and cloned between *Kpn*I and *Hind*III restriction sites of pTub5cat vector (Kim et al., 1993). A 2 kb genomic fragment of the 3′ flanking region (after the stop codon) has been amplified and cloned into the XbaI and *Sac*II site (Table S1).

2.3. Parasites transfection and selection of stable transformants

T. gondii tachyzoites (RH*hxgprt*-) transfections were undertaken by electroporation as previously described (Soldati and Boothroyd, 1993) using 80 μg pTub5CAT-5′-3′TgASP1 (*Pvu*I/*Pvu*I fragment). Twenty micrograms of chloramphenicol have been added to the culture medium to allow integration of the plasmid vector into *T. gondii* genome as previously described (Kim et al., 1993). Stables clones were isolated by limiting dilution in 96-well plates. These parasites clones were screened by PCR and RT-PCR for deletion of the endogenous TgASP1 gene using primers listed in Table S1. PCR products at the expected size were cloned and sequenced.

2.4. Immunofluorescence assay (IFA) and confocal microscopy

For the indirect immunofluorescence assay, RHhxgprt- and *Tgasp1*-tachyzoites were used to infect HFF cells that were growing on glass coverslips. After 24 h–36 h, cells were washed with PBS and were fixed with 4% paraformaldehyde in PBS or 4% paraformaldehyde/0.025% glutaraldehyde (PFA/GA) in PBS for 15 min and neutralized with PBS containing 0.1 M glycine for 5 min. Fixed cells were permeabilized for 20 min with 0.2% Triton-X100 in PBS and blocked with 2% BSA in PBS-Triton X-100 for 20 min. The cells were then stained for 1 h with primary antibodies followed by goat-anti-rabbit or goat-anti-mouse IgG conjugated to Alexa-Fluor-488 or Alexa-Fluor-594 (Molecular Probes, Invitrogen) as secondary antibodies. DAPI staining was performed with a concentration of 0.1 μg DAPI/ml PBS before mounting the slides in FluoromountG (Southern Biotech).

Transient tranfections were done with pPhil1-YFP (Gilk et al., 2006), pDLC-EGFP (Hu et al., 2006), pMORN-EGFP (Gubbels et al., 2006), and pGRASP-YFP (Pelletier et al., 2002) in RH and *Tgasp1*-strains. Co-localizations were done using anti-TgGAP45 antibodies as described previously with goat-anti-rabbit IgG conjugated Alexa-Fluor-594 as secondary antibodies.

Confocal images were collected with a Leica laser scanning confocal microscope (TCS-NT DM/IRB and SP2) using a 1003 Plan-Apo objective with NA 1.4. Single optical sections were recorded with an optimal pinhole of 1.0 (according to Leica instructions) and 16 times averaging. Stacks of sections were recorded at ~0.2 lm vertical steps and projected using the maximum projection tool.

2.5. Western blot

Freshly released tachyzoites were harvested, washed in PBS, solubilized directly in SDS-loading buffer, separated by electrophoresis in 10–12% polyacrylamide gels and transferred. Western blots were incubated with respective mouse monoclonal antibodies or rabbit polyclonal antisera in PBS, 0.05% Tween 20 and 5% non-fat milk powder. After washes, the membrane was incubated with a peroxidase-conjugated goat anti-mouse (SIGMA) or anti-rabbit antibody (Molecular Probes). Bound antibodies were visualized using the ECL plus system (GE Healthcare Bio-Sciences). Rabbit monoclonal anti-catalase (1:1000) was used as loading control.

2.6. Plaque assay

A host cell layer was infected with parasites (wild type or *Tgasp1*-) for 5 days before the cells were fixed PFA/GA. The host cell layer was then stained for 15 min at RT with Giemsa (Sigma–Aldrich) diluted 1:5 in dH_2O. Host cells were washed with water and mounted in Fluoromount G (Southern Biotech). Plaques were visualized under the microscope (2.5× objective).

2.7. Intracellular growth assay

Host cells were inoculated with freshly egressed parasites and incubated for 2 h before washing. Parasites were allowed to grow for 24 h before fixation with PFA/GA. Double immunofluorescence assays (IFAs) were performed using anti-actin (mouse) and anti-Gap45 (rabbit) antibodies. The parasites of at least 100 vacuoles were counted for each condition, and the results are representative of three independent experiments.

2.8. In vivo virulence analysis

To assess parasite virulence *in vivo*, groups of five female seven -weeks-old BALB/C mice were infected intraperitoneally with 100 tachyzoites of the wild type or *Tgasp1*-strains. The virulence was determined as the time necessary to kill the mice. The animals were inspected twice daily (AM and PM). When severe defects were observed, mice were killed. In parallel, by plaque assay we confirmed equal numbers of viable parasites were injected. The animal experiments were conducted following the approach and within the guidelines of the committee in (Veterinerian Geneva Cantonal Office; Project Licence: 1026/3450/2).