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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).

3. Results and discussion

3.1. Tgasp1 can be deleted by classical knock-out

The TgASP1 locus was disrupted by double crossover using Chloramphenicol AcetylTransferase (*cat*) as selection cassette, conferring the resistance to chloramphenicol (Kim et al., 1993). The pT5TgASP1/cat plasmid contains 1.9 kb and 2 kb of 5′ and 3′ flanking regions of *TgASP1* respectively and recombination at the *Tgasp1* locus is depicted in Fig. 1A. Specific PCR amplifications were used to discriminate between wild type (WT) and the knockout locus (*asp1-ko*) and to demonstrate the absence of the *TgASP1* Open Reading Frame (ORF) (Fig. 1B). RT-PCR was performed to confirm the absence of *TgASP1* transcripts (data not shown). Finally, the *asp1-ko* parasites were examined by Western blot to confirm the absence of TgASP1 and hence simultaneously the specificity of the anti-ASP1 antibodies. Two bands at the 35 kDa (intermediate processed form) and 30 kDa (mature final form) were detected on WT parasite lysates and disappeared in lysates from *asp1-ko* (Fig. 1C). By indirect immunofluorescence assay (IFA), the WT parasites showed a punctuate labelling at the apical pole with anti-TgASP1 antibodies as described previously (Shea et al., 2007), whereas no labelling was observed with *asp1-ko* parasites (Fig. 1D). The generation of *asp1-ko* parasites established that this protease is not indispensable the *T. gondii* tachyzoites proliferation in tissue culture.

3.2. TgASP1 is dispensable for T. gondii growth in vitro and in vivo

Further analysis revealed that the *asp1-ko* parasites were morphologically indistinguishable from WT parasites. The lytic cycle involves host cell invasion, intracellular growth and division and egress from the infected cells. A defect in any of these steps can be monitored by performing a plaque assay that monitors several lytic cycles over a period of 5 days on human foreskin fibroblasts (HFFs) that are then fixed and stained with Giemsa. No detectable difference in the size of the plaques was observed between WT and *asp1-ko*, indicating no obvious defect in the lytic cycle (Fig. 1E). To confirm the absence of intracellular growth defect, growth assays were performed by scoring the number of parasites per vacuole, 24 h after inoculation of the HFF. In accordance with the results obtained in plaque assays, the *asp1-ko* parasites showed no significant growth impairment compared to WT parasites (Fig. 1F). Lastly, we conducted an *in vivo* infection to monitor the virulence of *asp1-ko* parasites by infecting the mice with 100 parasites of either *asp1-ko* or WT parental strain. Both strains killed the mice within 7–8 days with the same kinetics (data not shown). A refined examination of the morphology of the nucleus and the organization, biogenesis and segregation of the endosymbiotic organelles (mitochondrion and apicoplast) revealed no defect (Fig. S2).

Given that TgASP1 was previously shown to reside in a novel compartment of the secretory system that potentially serves a link between the Golgi and the IMC (Shea et al., 2007), we investigated the localization of the cis-Golgi marker, GRASP by pYFP-GRASP transient transfection (Pelletier et al., 2002). No alteration of GRASP localization was observed (Fig. S2).

3.3. TgASP1 deletion has no effect on secretory organelles

Despite the absence of apparent phenotype in *asp1-ko*, we then focussed on potential TgASP1 substrates by studying the localization of different proteins and their processing in *asp1-ko* compared to the parental strain. Such substrates should accumulate as precursors in *asp1-ko* whereas their cognate cleaved products would be generated in WT parasites. Micronemes and rhoptries store proteins that have frequently undergone a processing step, though the action of maturases, during their trafficking to the secretory organelles (Carruthers, 2006). We first examined the trafficking and the processing of micronemal proteins that are eventually secreted at the anterior surface of the parasite and involved in host cell receptor attachment. *T. gondii* MIC2, MIC4 and MIC6 trafficked normally in *asp1-ko* and showed a typical intense staining of the apical half of tachyzoites (Fig. 2A). No alteration in level of expression or processing of micronemal proteins was detected (Fig. 2A). Rhoptries contain serine and cysteines proteases and ROP proteins that have been previously reported to be processed (Dubremetz, 2007). As with the micronemes, the localization of proteins in the rhoptry neck (RONs) and in the rhoptry bulb (ROPs) and their level of expression were unaffected in *asp1-ko* (Fig. 2A). Proteins stored in the dense granules (GRA) extensively modify the parasitophorous vacuole and are thought notably to participate in nutrient uptake from the host cell. Even though GRA proteins are generally not subject to proteolysis, we examined their localization and their expression in *asp1-ko*. As shown in Fig. 2A, GRA3 is detected as small dots within the parasite as well as in the vacuolar space as previously described (Bermudes et al., 1994). Taken together, these results indicate that TgASP1 plays no significant role in the processing of proteins destined to the secretory organelles.

3.4. TgASP1 deletion does not affect IMC biogenesis

Given that TgASP1 shows a punctuate localization associated with the secretory system in resting cells and relocalizes with the nascent IMC of dividing parasites (Shea et al., 2007), we scrutinized whether *asp1-ko* parasites exhibited any defect in IMC biogenesis, in the gliding machinery, or in the pellicle integrity. No change in the localization of IMC1 was observed at the periphery of mother cells and in daughter cells (Fig. 2B). Moreover TgMLC1 and TgGAP45 localized accurately to the pellicle in *asp1-ko* parasites (Fig. 2B) and the expression level of these proteins is unaltered (Fig 2B). These results suggest that neither the IMC nor the glideosome are affected by the absence of TgASP1.

We then compared the localization of two cytoskeleton proteins Phil1 and the dynein light chain (TgDLC) by transient transfections using pEGFP-TgDLC (Hu et al., 2006) and pPhil1-YFP vectors (Gilk et al., 2006). As described previously, TgPhil1 localizes to the parasite periphery, concentrated at the apical pole just basal to the conoid in both strains (Fig. S3). TgDLC localized to a part of the IMC described as the apical cap in the two strains (data not shown). TgASP1 accumulates in the nascent daughter cells in close proximity to the TgMORN1 ring at the base of forming daughter cell IMCs (Gubbels et al., 2006; Heaslip et al., 2010). TgMORN1 has recently been shown to be the dynamic key organizer for the basal complex (Heaslip et al., 2010) and the redistribution of the protein during parasite division does not appear to be altered in *asp1-ko* (Fig. S3).

Finally, a global strategy by Difference Gel Electrophoresis (DIGE, Nelson et al., 2008) was used to search for substrates of TgASP1 by comparing lysates from wild type and *asp1-ko* cell lines but this approach also failed to detect any significant and reproducible difference between the two strains (data not shown).

In conclusion, the detailed phenotypic investigation of *asp1-ko* failed to reveal any significant defect or to identify a substrate for this protease. In contrast attempts to disrupt other TgASPs failed so far suggesting that some members of this class of proteases might play a more predominant role in *T. gondii*. The non-vital function of TgASP1 could be explained by a redundancy between the ASPs, however this hypothesis is not very plausible based on the phylogeny and the distinct subcellular localizations of these proteases. The rhomboid like protease TgROM1 and cysteine protease TgCPL are other examples of dispensable enzymes in *T. gondii* for which the deletion conferred very modest effects on tachyzoites (Brossier et al., 2008; Larson et al., 2009). A more prominent role for these proteases in other life stages can be envisioned and indeed TgASP1

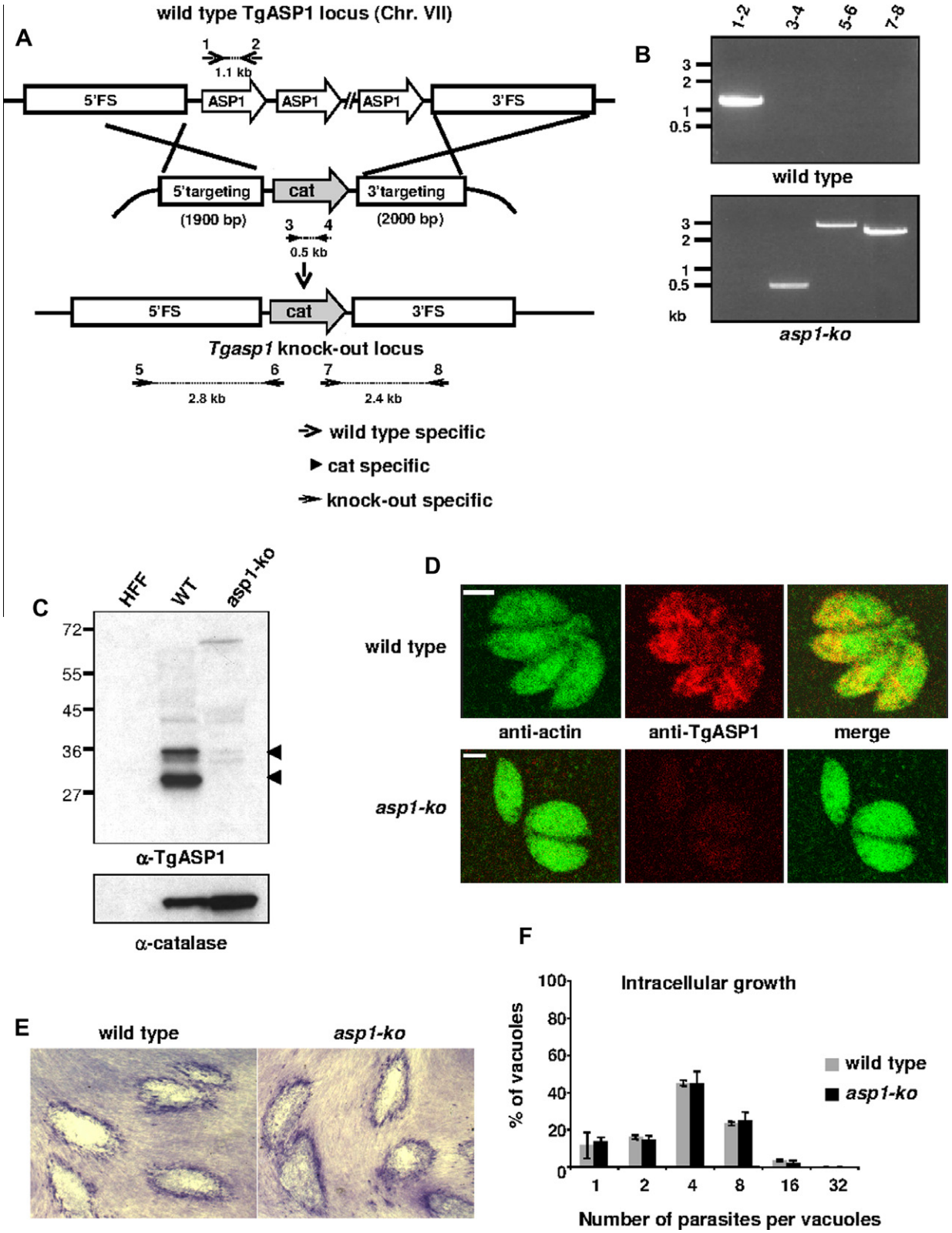

Fig. 1. Targeted disruption of *TgASP1* gene and growth phenotype *in vitro*. (A) Schematic representation of *TgASP1* modified locus. The coding sequence of ASP1 has been replaced by the chloramphenicol acetyltransferase (*cat*) resistance marker by double crossover. The primers used for analytical PCR are indicated by arrows. The size of the expected fragments shown as lines is indicated. (B) Disruption of *Tgasp1* gene was confirmed by genomic PCR analysis on the locus of WT and clonal asp1-ko parasites. Numbers on the top of each lane indicate the respective primer combination used in each PCR reaction. (C) Western blot analysis of WT and asp1-ko parasites to check for the absence of TgASP1 protein in asp1-ko parasites using anti-TgASP1 antibodies. The arrows indicate two forms of TgASP1: an intermediate processed form (35 kDa) and the final mature form (30 kDa). (D) Indirect immunofluorescence assay on HFF infected with WT or *asp1-ko*. Rabbit polyclonal antibodies specific to TgASP1 (in red) show no staining on *asp1-ko*. Control staining of parasites is shown with anti-actin (in green). (E) Plaque assays were performed by incubating host cells with WT or *asp1-ko* parasites for 5 days and staining with Giemsa. (F) For intracellular growth assay, WT or *asp1-ko* parasites were grown during 24 h and the number of parasites per vacuole (*x*-axis) were counted. At least 100 vacuoles were scored for each condition and the results are representative of three independent experiments. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this paper.)

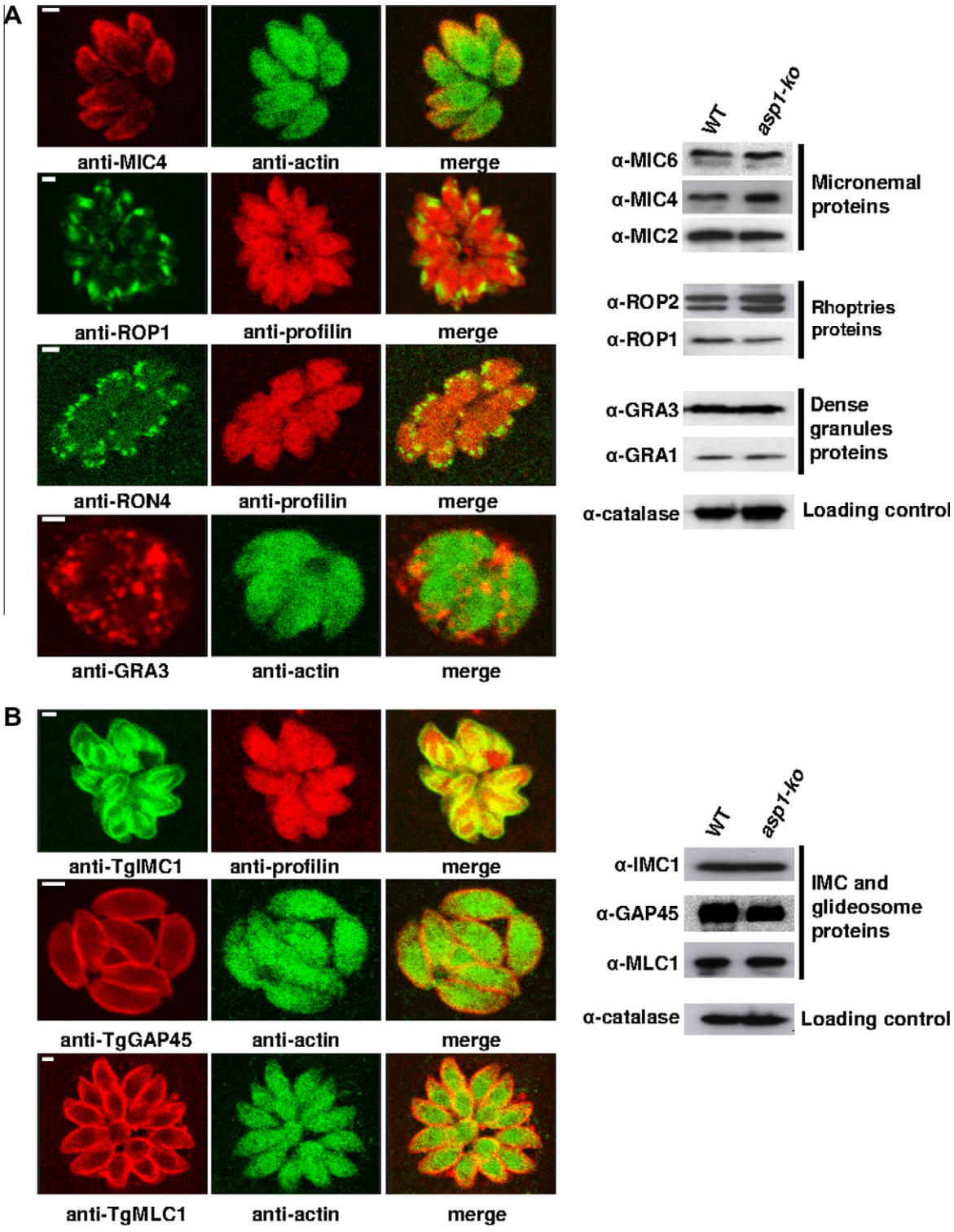

Fig. 2. Deletion of *TgASP1* has no impact on the content and processing of secretory organelles contents or on the IMC and glideosome assembly. IFA were performed using anti-MIC4, anti-ROP1, anti-RON4 and anti-GRA3 antibodies (A) and anti-IMC1, anti-GAP45 and anti-MLC1 antibodies (B). Co-localizations were performed with anti-actin or anti-profilin (Plattner et al., 2008). Scale bar: 2 μm. The level of expression and processing of the proteins examined are not affected as monitored by western blot on WT and *asp1-ko* parasites.

is expressed in both tachyzoites and bradyzoites (Shea et al., 2007). The presence and conservation of ASP1 in *T. gondii* and *N. caninum* has a "raison d'etre". The spectra of analyses performed in this study did not lead to the identification of its role and hence further investigations and experimental conditions are required to unravel the function and substrate(s) of this protease.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.exppara.2011.05.003.

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